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SOURCES OF ENERGY FOR AZOTOBACTER, WITH SPECIAL REFERENCE TO FATTY ACIDS[1]

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Introduction

The thermo-chemical phenomena involved in the fixation of free nitrogen by various micro-organisms are not well understood. It has been assumed that the fixation process is endothermic in nature and that the necessary energy is, in the case of the *Azotobacter* group of organisms, derived from the oxidation of organic compounds, principally of a carbohydrate, acid, or alcohol nature.

Regardless of whether the initial process through which nitrogen is brought into combination is exo- or endo-thermic, no one has been able to establish definitely a measurable fixation of nitrogen by *Azotobacter*, or any other nitrogen-fixing group of organisms, in the complete absence of some form of organic matter. Furthermore, growth and nitrogen fixation have been found to run more or less parallel with the quantity and nature of the organic material available, provided the material is non-nitrogenous in nature. It may be assumed safely, therefore, that an organic food material of some kind is essential in the metabolism of this group of organisms. This being true, it would seem highly desirable, both from a theoretical and practical standpoint, to secure as much information as possible relative

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to the different organic food substances suitable for these organisms and also the relative efficiency with which different compounds can be used.

As an aid in the solution of some of the more complicated thermo-chemical questions involved it seemed desirable to ascertain, if possible, whether any quantitative relationship existed between the potential energy content of the organic material utilized, on the one hand, and the quantity of growth and nitrogen fixed, on the other. It was with the hope of securing information along these lines that this work was undertaken.

More specifically this investigation has been concerned with seeking an answer to the following questions: (1) Is there any difference in the relative availability of the lower fatty acids as a source of carbon, or organic food substance, for *Azotobacter*? (2) If *Azotobacter* exhibits differences in ability to utilize different fatty acids, can such differences be associated with the structure, size, or energy content of the molecule?

The following criteria were used in judging the ability of *Azotobacter* to utilize the various acids: (a) the variation in visible growth; (b) the disappearance of the acid; (c) the fixation of nitrogen; (d) changes in the hydrogen-ion concentration of the medium.

The lower fatty acids were selected for study because they compose a series of compounds exhibiting many characteristics in common, yet in the series the molecule increases in definite increments from fairly simple to fairly complex. Furthermore, the heat of combustion of these compounds increases directly as the molecular weight increases. By including the iso compounds, two molecules varying in structure but identical in composition and energy content could be compared. An additional desirable characteristic possessed by this series of compounds is that they are found as free acids or as constituents of fats in nature, and some of them are already known to serve as organic food for *Azotobacter*. One other characteristic essential in a series of compounds suitable for a study of this nature is susceptibility to fairly easy quantitative analysis. Not many series of organic compounds of which the members possess the

desirable characteristics enumerated above can be found, and possibly there is no other series in which as many members may be used so advantageously. Unfortunately, lack of solubility prevents any quantitative use of members of this series above six carbon atoms, but even then there would seem to be enough members of the series that can be used to give valuable information if carefully studied.

Methods

General procedure—The general procedure has been to prepare, in one batch, as carefully as possible, all the medium necessary for a single experiment. Measured quantities of this were placed in the culture flasks which were then stoppered with cotton and sterilized in the autoclave. After sterilization the flasks were inoculated as uniformly as possible with a heavy suspension of organisms washed from the surface of mannitol-soil-extract agar upon which active vigorous growth was taking place. No old or apparently non-vigorous growing culture was used as an inoculum. After varying periods of incubation the cultures were removed from the incubator and the qualitative and quantitative analyses were made as indicated.

Where supplementary aeration was resorted to, an inlet tube of glass, containing five to seven small openings near the end, was inserted in a rubber stopper in such a way that when the stopper was tight in the culture flask the end of the tube almost reached the bottom of the flask. The stopper was also provided with an outlet tube to be connected to a vacuum system. The stopper and connecting tubes were sterilized separately and inserted after inoculation. Cotton was forced into the ends of the connecting tubes as a precaution against possible contamination. In the aerated experiments 300-cc. Pyrex Erlenmeyer flasks were used as culture containers, and all those in any one experiment were connected in series so that the same quantity of aeration was provided for all. While incubating, a vigorous bubbling of air through the media was continuously maintained. Before entering the first culture flask the air was washed through flasks arranged as mentioned above, of acid, alkali, and water. Despite special precautions to prevent contamination there was one type of foreign organism difficult to keep out.

Where no special aeration was provided the cultures consisted of 50 cc. of media in 300-cc. flasks; 100 cc. media in 750-cc. flasks; or 200 cc. media in 1000-cc. Pyrex Erlenmeyer flasks. These quantities of media in the flasks indicated always exhibited a large surface area compared to the depth, and while aeration was certainly not as vigorous as where air was drawn through the culture, nevertheless it was ample for very rapid growth. Growth at the bottom of the culture was frequently observed before it made its appearance on the surface, indicating aeration throughout the culture. These cultures were left stationary except when being handled for examination, and even then care was taken not to shake so vigorously as to break up any film that might be forming on the surface.

Medium—Unless otherwise stated the medium used in the various experiments had the following composition, and its suitability is evidenced by the very rapid growth that took place when the organic material was assimilable:

K_2HPO_4	2.50	gms.
$MgSO_4$	.20	gm.
NaCl	.20	gm.
$CaCl_2$	.05	gm.
$FeCl_3$ (10 per cent sol.)	1	drop
Organic material	1	per cent
Distilled water	1000	cc.

In some of the preliminary experiments only 0.50 gm. of K_2HPO_4 was used, but it was observed that when such a small quantity of phosphate was added the hydrogen-ion concentration sometimes changed so rapidly and markedly that growth was very soon inhibited by the increase of hydroxyl-ions. A rapid increase in hydroxyl-ion concentration was always observed when large quantities of a metallic salt of an organic acid were metabolized, and probably arose from the formation of an hydroxide by the metallic-ions set free when the acid radicle was assimilated by the organisms.

Even with 2.5 gms. phosphate and an excess of $CaCO_3$ the buffering effect was barely sufficient to permit of complete oxidation of 1.0 per cent acid. In fact, in some instances there is evidence to indicate that the high alkalinity accompanying vigorous oxidation not only prevented further activity, as is

evidenced by the failure of the organisms in certain cultures to oxidize all the acid even where abundant growth occurred, but actually resulted in the death of most or all of the organisms present. In case of some of the less soluble acids the quantity added was only 0.5 per cent.

When the organic material was fatty acid it was, in all except a few preliminary experiments, added to the entire volume of distilled water. An excess of $CaCO_3$ was then added, and the material boiled until the reaction became neutral to brom-thymol-blue, indicating complete transformation into the calcium salt, after which it was filtered. This procedure was resorted to in order to hasten the completion of the reaction between the weakly dissociated acid and calcium carbonate. The other salts were then added, and if any change in the reaction took place it was again adjusted to neutrality by the addition of sodium hydroxide or sulphuric acid as needed. The medium was then measured into the culture flasks, care being taken to keep it well agitated in order to secure an equal distribution of the precipitate, a small quantity of $CaCO_3$ added, the flask plugged with cotton and sterilized in the autoclave at ten pounds pressure. The final reaction of medium prepared as indicated was never far from P_H 7.0.

Obviously, one could not depend upon the original weight of any organic material subjected to the manipulations described in the preceding paragraph as indicating the final concentration. It was necessary, therefore, to prepare controls and make quantitative analyses of the final concentration of the organic materials in all cases.

The agar used for maintaining stock cultures, for testing the purity of cultures, and for the preparation of the inoculum was a soil-extract-mannitol agar prepared as follows: One thousand gms. fertile garden soil were added to 1000 cc. distilled water and subjected to fifteen pounds pressure in the autoclave for thirty minutes, after which $CaCO_3$ was added and the mixture filtered. The clear filtrate was made up to 1000 cc. with distilled water. To 900 cc. distilled water was added 100 cc. soil extract, 0.5 gm. K_2HPO_4, 10 gms. mannitol, and 15 gms. agar agar. After heating in the autoclave to bring the agar agar into

solution, and while still hot, phenolphthalein and sufficient sodium hydroxide were added to give a distinct pink color.

Cultures—The following include all cultures employed in any experiment, together with their origin. They were selected from among more than one hundred available cultures of *Azotobacter*. Those strains that were used to any appreciable extent were selected primarily because of their vigorous growing and nitrogen-fixing ability. Cultures Nos. 3a and 3b were strains of *Azotobacter chroococcum* secured from S. A. Waksman, of New Brunswick, N. J. Cultures Nos. 4, 5a, 5b, 6, 7, 8, 57, 58, 59, 60, 62, and 66 were isolated from different Colorado soils in the laboratory of W. G. Sackett, Fort Collins, Colo. Culture No. 94 was a strain of *Azotobacter vinelandii* from the Bureau of Plant Industry, U. S. Dept. Agr., Washington, D. C. Culture No. II was received from W. Omeliansky, Leningrad, Russia. Culture No. 218, a strain of *Azotobacter chroococcum* marked "K," was received from Chr. Barthel, Stockholm, Sweden. Cultures "C" and "R" were received from the Rothamsted Experiment Station, England. "C" came originally from a single cell strain of H. R. Christensen's and "R" was isolated from soil. Cultures Nos. 178, 187, 188, 194, and 165 were all isolated in this laboratory from the following soils, respectively: No. 178, Gloucester loam from Minnesota; No. 187, field soil from New York; No. 188, cotton and sugar cane soil, Virgin Islands; No. 194, soil from V. L. Winogradsky, Paris, France; No. 165, irrigated potato field soil from Wyoming. The only unidentified strain used to any appreciable extent was No. 62. This culture possessed the characteristics of *Azotobacter chroococcum* in that it grew abundantly as grayish-white opaque, distinct colonies, soon turning brown and eventually black with a more or less wrinkled dry surface.

Before any culture was used in any experiment it was carefully tested for purity by repeated streaking and re-isolation from individual, microscopically examined colonies, until assured of the presence of only one type of organism. Furthermore, after incubation most cultures were again examined for purity and if evidence of contamination was present it has been so recorded.

Inoculum.—The inoculum was prepared by streaking the

entire surface of a Kolle flask of soil-extract-mannitol agar with the desired culture, incubating 48–96 hours, or until the entire surface was covered with a uniform thick growth, and suspending this growth in 25–50 cc. of sterile water. This gave a suspension of such density as to be practically opaque in a depth of only half an inch. One or two per cent of this was used as the inoculum, thus insuring a very heavy inoculation.

Incubation.—All cultures were incubated either at summer room temperature or in an incubator at 28–32° C. Room temperature was quite favorable in the summer but during the winter the temperature dropped too low at night for active growth. Most of the aerated experiments were run at room temperature, while all non-aerated experiments were incubated at 28–32° C.

CHEMICAL METHODS

Dextrose.—Quantitative dextrose determinations were made by the Shaffer-Hartmann ('21) iodometric method. Where a heavy growth of *Azotobacter* had taken place the slime-like material present was precipitated by adding 1.0 per cent of a mixture of 2.5 gms. phosphotungstic acid and 5.0 gms. H_2SO_4 before sugar determinations were made. Preliminary experiments proved that sugar could be recovered quantitatively when added to a culture and treated by this method.

Total nitrogen.—The Gunning modification of the Kjeldahl method was employed for total nitrogen. If the culture consisted of only 50 cc. of medium the entire volume was utilized, otherwise after making the culture up to the original volume 25-cc., or more frequently 50-cc., duplicated samples were run. A small piece of copper wire, 7 gms. of anhydrous sodium sulphate, and 35 cc. H_2SO_4 were added and digestion continued for one hour after the solution became clear (see Latshaw, '16). Table 1 indicates the degree of accuracy with which duplicate determinations checked. In some of the experiments where aeration was employed the data for nitrogen determinations did not seem conclusive, and it has been thought best to leave them out entirely. No significance is attached to an increase in nitrogen of less than 0.5 mgs. per 100 cc. of medium, and all the data recorded in the tables are based upon 100 cc.

TABLE I

ACCURACY WITH WHICH TOTAL NITROGEN DETERMINATIONS CHECKED

Sample No.	Mgs. nitrogen recovered from		
	Peptone solution	Peptone solution	Azotobacter culture
1	9.58	9.61	2.21
2	9.52	9.52	2.34
3	9.61	9.52	2.14
4	9.58	9.58	2.21
5	9.52	9.52	2.08
6	9.52	9.58	2.27
Average	9.55	9.55	2.25

Volatile acid.—Practically all the acids used were Eastman Kodak Co. products. Quantitative determinations have been made by distilling 100 cc. from a total volume of 110 cc. Pyrex Erlenmeyer flasks of 300 cc. capacity connected to Liebig condensers and surrounded by an asbestos shield were used as distillation flasks. These were heated by an electric hot plate, and it required 30–45 minutes, depending upon the acid, to distil over 100 cc. Titrations were made in increments of 20 cc. unless it had previously been noted that practically all acid had disappeared, in which case the entire 100 cc. were titrated at one time. This fractional titration was employed in order to enable the plotting of the titration curves to detect the transformation of a higher into a lower acid. Phenolphthalein was employed as an indicator, and care was exercised that all vessels and wash water were neutralized before being used.

Figures 1 and 2 are given to show the relative titration curves of the different normal acids and also to show that there was no indication of an acid with a higher molecular weight being transformed into one of lower molecular weight. The curves for the standard acid solution and for the cultures in which abundant growth had taken place coincide as well as would curves from two different batches of acid. The culture distillation curves are from cultures in which approximately half the original acid had disappeared. Curves for iso compounds would show the same thing. These curves are plotted on a basis of the per cent of the total recovered that came over in each 20-cc. fraction, when 100 cc. were distilled from a total volume of 110 cc.

Calculations of the quantity of acid present were based upon quantitative distillations of carefully standardized acids distilled from pure water to which a small quantity of sulphuric acid had also been added. The data in table II show the per cent

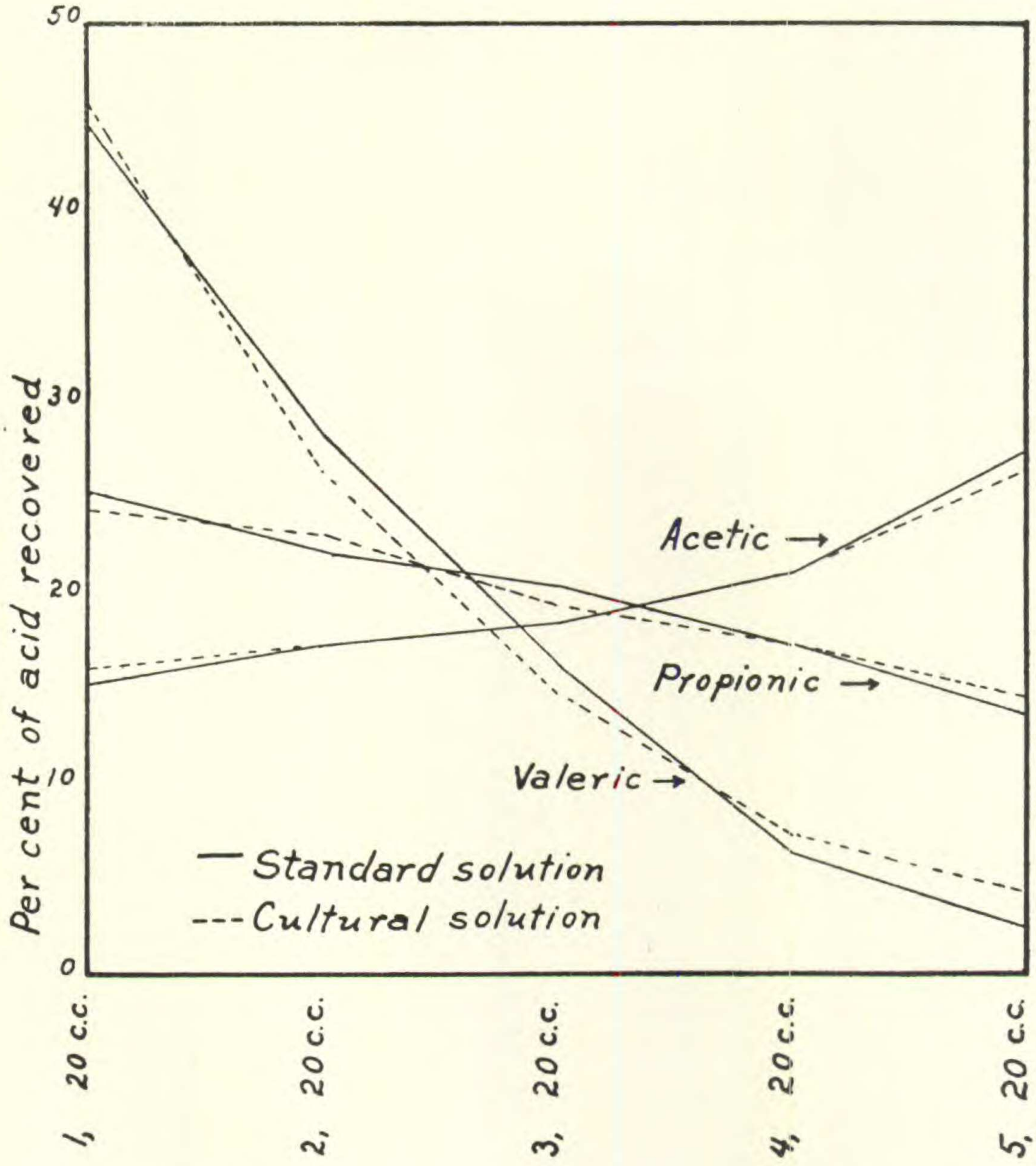

Fig. 1. Distillation curves for volatile acids from standard solutions and from cultures in which approximately half of the acid had been metabolized.

of the total recovered from the different acids when varying quantities up to 100 cc. of the total 110 cc. had been distilled. Data recorded in table III show the degree of accuracy with which triplicate determinations checked. The figures in table

IV indicate that the presence of the other constituents of the culture medium did not interfere with the recovery of the volatile acid. The quantity of medium distilled was, unless indicated to the contrary, 25 cc., and duplicate or triplicate samples were always run. Only averages of the two or three checks are

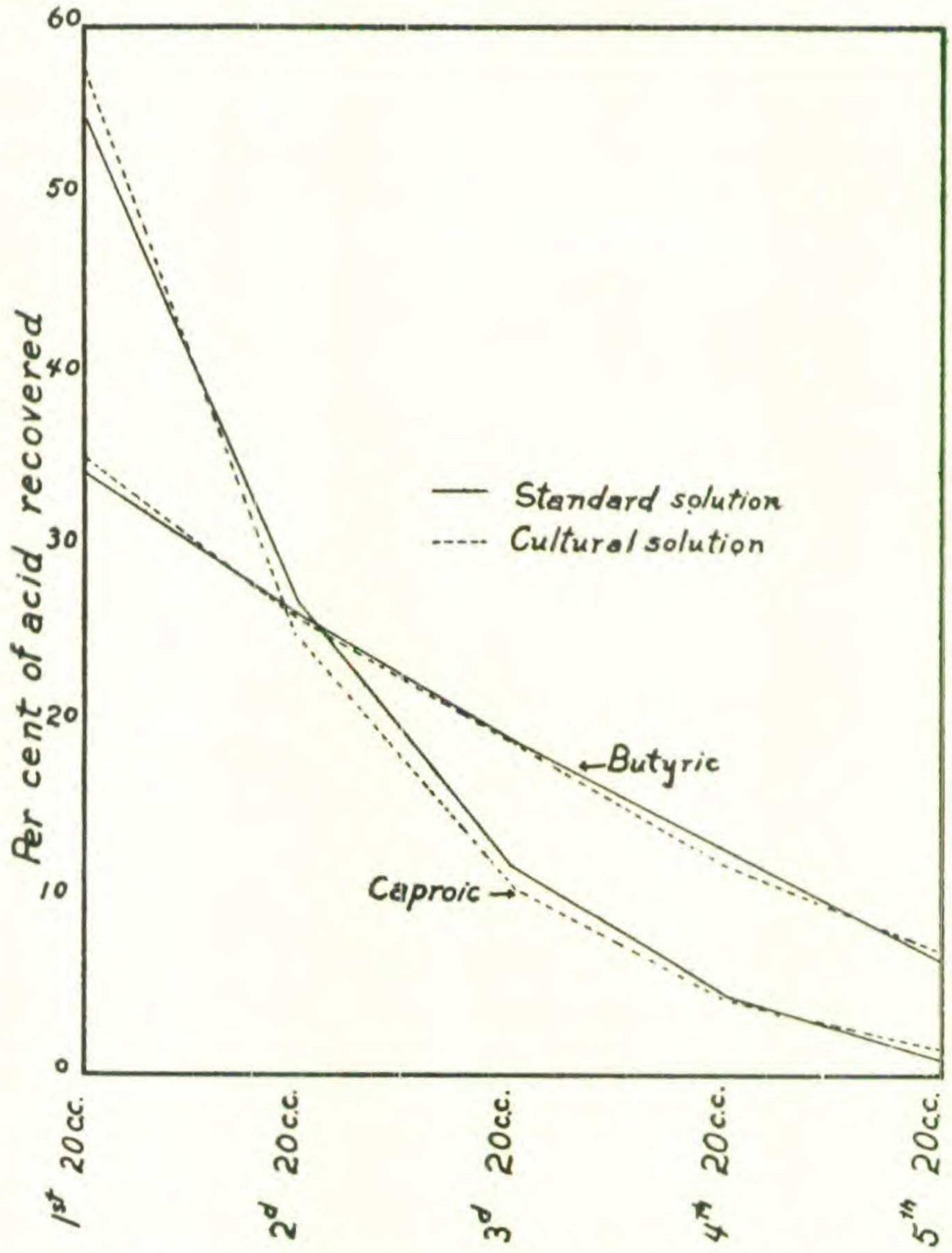

Fig. 2. Distillation curves for volatile acids from standard solutions and from cultures in which approximately half of the acid had been metabolized.

recorded. When distilled from the culture medium the volatile acid was freed from calcium by adding an excess of sulphuric acid. The CO_2 thereby liberated was removed by aerating vigorously for thirty minutes.

In the aerated cultures, run for the longer periods of time,

TABLE II

RECOVERY OF VARIOUS VOLATILE ACIDS WHEN 100 CC. WERE DISTILLED FROM A TOTAL OF 110 CC.

Cc. distilled	Per cent recovered									
	Formic	Acetic	Propionic	Normal butyric	Iso-butyric	Normal valeric	Mono-hydrated valeric	Tri-hydrated valeric	Normal caproic	Iso-caproic
20	7.3	12.5	24.2	33.5	42.7	43.9	45.5	50.7	60.4	57.8
40	15.9	26.2	45.5	59.2	70.6	71.8	72.2	77.1	88.4	84.5
60	26.2	41.3	64.9	78.0	87.5	87.9	86.2	89.7	100.0	95.6
80	40.0	58.5	81.2	90.5	95.5	94.8	93.2	97.8	100.0	100.0
100	62.2	80.8	94.4	97.2	98.9	97.4	96.1	99.4	100.0	100.0

TABLE III

THE DEGREE OF ACCURACY WITH WHICH TRIPLICATE DISTILLATIONS OF VARIOUS ACIDS COULD BE MADE

Cc. distilled	Per cent of acid recovered															
	Formic				Acetic				Propionic				Valeric			
	Flask 1	Flask 2	Flask 3	Ave.	Flask 1	Flask 2	Flask 3	Ave.	Flask 1	Flask 2	Flask 3	Ave.	Flask 1	Flask 2	Flask 3	Ave.
20	8.00	8.30	8.00	8.10	12.50	12.60	12.60	12.57	21.10	21.10	21.10	21.10	50.20	50.40	50.00	50.20
40	16.70	17.10	16.70	16.23	26.00	26.10	26.20	26.10	42.80	44.10	42.60	43.12	75.40	78.10	78.10	77.20
60	27.40	27.60	27.40	27.46	40.70	40.70	40.70	40.70	62.10	61.60	61.50	61.73	90.80	91.90	91.70	91.47
80	41.10	41.40	41.10	41.20	57.50	57.50	57.40	57.47	78.60	78.20	78.60	78.47	96.30	96.90	96.90	96.70
100	61.60	61.90	61.60	61.70	78.80	77.90	78.60	78.43	93.40	93.40	93.60	93.47	100.00	98.70	98.50	99.07

TABLE IV

RECOVERY OF ACIDS FROM PURE WATER SOLUTION AND FROM AN AZOTOBACTER CULTURE MEDIUM; EXPRESSED IN EQUIVALENT QUANTITIES OF N/20 NaOH

Cc. distilled	Butyric		Valeric		Caproic		Propionic	
	H_2O	Medium	H_2O	Medium	H_2O	Medium	H_2O	Medium
20	8.40	8.45	11.40	11.60	9.25	9.45	5.95	5.95
40	14.70	14.95	17.60	17.90	13.80	14.00	11.60	11.95
60	19.50	19.80	20.85	20.95	15.82	16.00	16.60	16.65
80	22.80	23.10	22.10	22.15	16.55	16.70	21.10	21.20
100	24.65	24.80	22.56	22.50	16.75	16.85	25.00	25.10
Total present	25.25	25.45	22.80	23.00	16.90	17.05	26.90	27.10
Per cent recovered	97.60	97.50	98.90	98.00	99.10	98.80	92.90	92.60

there were indications in some instances that slight losses of acid occurred. This is not surprising, though, in view of the fact that despite four wash-bottles, designed to remove any acids and bases that might be present in the laboratory air as well as saturate the air with moisture, there were sometimes rather large losses in the volume of culture medium. Such losses were no doubt principally due to evaporation but probably in a less degree to the mechanical removal of moisture due to the bursting of so many bubbles. Owing to such discrepancies it has been difficult to evaluate accurately the utilization of acid by the growing cultures in certain instances. We have therefore recorded as questionable such losses in the aerated experiments unless they obviously exceeded such losses where observations were possible.

Qualitative reaction.—It has been thought best to check roughly (time would not permit accurate determination) the hydrogen-ion concentration of the cultures in order to determine if the reaction were suitable for growth. For this purpose use has been made of brom-cresol-purple, brom-thymol-blue, phenol-red, cresol-red, phenolphthalein, and thymol-blue. Since the medium was only tested roughly as to whether it was acid, alkaline, or neutral to those indicators within whose range its reaction lay, the figures recorded in the various tables are merely approximate. Only in those instances where the medium was found to be alkaline to thymol-blue and is recorded as 9.0 + has there been any indication that the reaction was unfavorable. In all

probability a medium alkaline to thymol-blue has an unfavorable, if not toxic, effect upon *Azotobacter* (see Johnson and Lipman, '22).

Preliminary Experiments

A large number of experiments of a preliminary nature have been performed. In fact, before any method, or step in a method, or culture was adopted for experimental use it was carefully tried to see that it would work satisfactorily. Among these preliminary experiments there are, aside from those already mentioned, a number that seem to be of sufficient significance to record.

Experiments reported by Hunter ('23) indicated that by drawing a current of air through the medium, growth of *Azotobacter* and fixation of nitrogen could be greatly stimulated. Since the use of any method that would hasten growth seemed desirable, in view of the slow assimilation of certain of the fatty acids reported by Mockeridge ('15), it was thought that Hunter's method might be used advantageously. Therefore, an experiment was designed not only to confirm Hunter's results but at the same time to determine whether varying the rate of aeration would influence quantitatively the consumption of the organic food and the fixation of nitrogen. The results of such an experiment are reported in table v.

No method of measuring quantitatively the rate of flow of air through the medium was available; however, in the samples subjected to "slow" aeration a slow continuous flow of bubbles, perhaps one a second, was maintained. In the "medium" aerated cultures the air was drawn through at least ten times as rapidly, while the cultures subjected to "rapid" aeration probably received ten times as much air as the "medium" aerated cultures.

The data show very definitely that increasing the rate of aeration increases both the rate of dextrose consumption and nitrogen fixation. There is some indication that the dextrose may possibly be utilized somewhat more efficiently with limited aeration, the average nitrogen-dextrose ratio being 1 : 79 for the "slow" aerated samples, whereas the corresponding ratio for the other samples was 1 : 115 and 1:113 respectively.

TABLE V

EFFECT OF AERATION UPON THE UTILIZATION OF DEXTROSE AND THE FIXATION OF NITROGEN BY CULTURE NO. 3A. INCUBATION 4 DAYS

Flask No.	Aeration "slow"			Aeration "medium"			Aeration "rapid"		
	Mgs. dextrose consumed	Mgs. nitrogen fixed	Mgs. dextrose per mg. nitrogen fixed	Mgs. dextrose consumed	Mgs. nitrogen fixed	Mgs. dextrose per mg. nitrogen fixed	Mgs. dextrose consumed	Mgs. nitrogen fixed	Mgs. dextrose per mg. nitrogen fixed
1	104	1.38	75	622	5.37	116	954	8.82	108
2	108	1.24	87	693	5.64	123	954	8.41	113
3	108	1.52	71	606	4.82	126	954	8.27	115
4	96	1.38	70	690	6.85	101	954	8.27	115
5	112	1.24	90	412	3.45	119	954	8.41	113
Average	105	1.35	79	605	5.25	115	954	8.44	113

In this connection it might be well to comment, in passing, upon the use of the term "dextrose-nitrogen ratio." Certain investigators in speaking of this relationship have made use of the term "carbon-nitrogen ratio." This term, it is believed, does not accurately express the relationship. If it is a question of the organism securing or setting free a certain quantity of energy per unit of carbon, as is usually considered, obviously the expression C : N ratio is incorrect in that the energy freed per unit of carbon depends upon the other elements combined with the carbon as well as upon the carbon. For example, caproic acid contains approximately twice as much energy per gram of material and one and one-fourth times as much energy per gram of carbon as does dextrose, both being six carbon atom compounds. It would seem, therefore, that it would be much more logical to express this relationship upon an energy or molecular basis.

In an effort to secure a desirable culture with which to carry out the more extensive investigations recorded in the next part of this paper, the experiments reported in tables VI and VII were designed. All the cultures available at that time were included in these tests.

TABLE VI

VARIATION IN UTILIZATION OF DEXTROSE AND FIXATION OF NITROGEN BY DIFFERENT CULTURES OF AZOTOBACTER

Culture No.	Incubation 2 days			Incubation 5 days		
	Mgs. dextrose consumed	Mgs. nitrogen fixed	Mgs. dextrose per mg. nitrogen fixed	Mgs. dextrose consumed	Mgs. nitrogen fixed	Mgs. dextrose per mg. nitrogen fixed
3a	169	2.20	77	620	4.96	125
3b	197	2.48	79	674	6.06	111
4	171	1.38	124	522	1.10	474
5a	179	2.06	82	679	3.58	189
5b	169	2.34	72	729	3.58	203
6	247	2.34	106	692	3.04	227
7	157	.82	192	250	.82	610
8	148	1.38	107	368	1.66	222

These data indicate that culture No. 3a was approximately as efficient in fixing nitrogen as any other. At the same time

TABLE VII

UTILIZATION OF DEXTROSE AND NORMAL BUTYRIC ACID* AND FIXATION OF NITROGEN BY DIFFERENT CULTURES OF AZOTOBACTER

Culture No.	Mgs. dextrose utilized	Mgs. nitrogen fixed	Mgs. dextrose used per mg. nitrogen fixed	Mgs. butyric acid utilized	Mgs. nitrogen fixed	Mgs. butyric acid used per mg. nitrogen fixed
3a	957	5.40	177	99	1.38	72
3b	957	4.55	210	98	1.76	55
4	84	.50	168	3	.00	—
5a	957	5.40	177	96	1.65	58
5b	957	6.20	154	99	1.24	80
6	620	3.02	208	99	1.38	72
7	84	.28	300	0	.00	—
8	957	3.44	284	99	.82	121

* Butyric acid neutralized with sodium hydroxide.

it grew abundantly, thus enabling ready detection of growth. It was also known to be a strain of *Azotobacter chroococcum*. For these reasons it was temporarily selected for further study.

These data also indicate that certain cultures (Nos. 4 and 7), to all appearance *Azotobacter*, developed very poorly in the dextrose and not at all in the butyric acid medium. When measured by the quantity of nitrogen fixed per unit of organic material used, the actively growing cultures were apparently capable of utilizing butyric acid much more effectively than dextrose. In this particular experiment the increased effectiveness with which butyric acid was used might have been due to the relatively low per cent present, compared with dextrose. It has been frequently observed that in the presence of small quantities of organic material a higher fixation of nitrogen takes place per unit of organic material consumed. In this experiment only 0.1 per cent butyric acid was present.

Having selected culture No. 3a the next step was to test it in a preliminary way with several acids. Accordingly, the experiment recorded in table VIII was arranged. For some unknown reason this culture was only able to utilize acetic acid among those tested. Similar results were secured in other experiments.

Other cultures having been added to our collection, further qualitative tests were carried out, among which was the protocol

TABLE VIII

UTILIZATION OF VARIOUS ACIDS BY CULTURE NO. 3A

Acid	Mgs. recovered per 100 cc. culture solution					
	Controls		Incubation period in days			
	Unaerated	Aerated	2	5	9	13
Formic	644	696	716	659	635	673
Acetic	1026	977	982	431	394	264
Propionic	1051	——	998	996	1002	996
Butyric	1018	1001	994	984	997	1001
Valeric	920	896	896	884	875	884
Dextrose	934	954	690	423	000	000

arranged in table IX. This experiment was also designed to gain some information relative to the effect of the cation as well as the acid radicle. The salts included were the only ones available of those particular acids at that time.

The results presented in table IX indicate very strongly that the cation is probably of as much significance in determining the availability of an acid as is the anion. None of the formates permitted growth. This has been characteristic of formic acid in all tests conducted with it and is probably due to the formation of formaldehyde. Aluminum acetate appears to be the most readily available salt of acetic acid tested, all the strains being able to assimilate it readily. Uranium acetate, on the other hand, was not assimilated by any of the cultures. Between aluminum and uranium lay calcium, ammonium, magnesium, potassium, and sodium salts, their availability being approximately in the order given.

Ammonium acetate, while apparently assimilated by all ten cultures, supported vigorous growth only in two instances. It is possible that when supplied with nitrogen the very small quantities of organic impurities finding their way into the cultures enabled the various cultures to make perceptible growth. This, though, does not seem probable. Only half the cultures were capable of making visible growth when the sodium salt was the only source of organic matter supplied, and only one out of the ten assimilated it readily. Culture No. 60 could not even metabolize dextrose readily.

This experiment, as well as others reported in this paper,

TABLE IX

UTILIZATION OF VARIOUS SALTS OF FORMIC AND ACETIC ACIDS BY DIFFERENT CULTURES OF AZOTOBACTER. INCUBATED 48 DAYS

Culture No.	Ammonium formate	Calcium formate	Sodium formate	Ammonium acetate	Aluminum acetate	Calcium acetate	Magnesium acetate	Potassium acetate	Sodium acetate	Uranium acetate	Dextrose
3a	—*	—	—	+	+++	+++	+	+	+	—	+++
4	—	—	—	+	+++	+++	?	—	—	—	+++
5a	—	—	—	+++	+++	+++	+++	+	+	—	+++
6	—	—	—	+	+++	+++	+++	+	+	—	+++
57	—	—	—	+++	+++	+++	+++	+	—	—	++
58a	—	—	—	+	+++	+	?	?	—	—	+
59	—	—	—	+	+++	+++	+	+	+	—	+++
60	—	—	—	+	+++	?	?	?	—	—	—
62	—	—	—	+	+++	+++	+++	+	+++	—	++
66	—	—	—	+	+++	+++	?	—	—	—	++

* In this and all succeeding tables a minus sign indicates no growth; a question mark indicates questionable growth; while the number of plus signs indicate the relative growth observed in that particular experiment. (The same number of plus signs indicate more or less comparable growth in different experiments.)

indicates a very wide variability in the metabolism of organisms belonging to the genus *Azotobacter*. The data also emphasize the great need for more specific physiological studies of this very interesting group of organisms. It would appear utterly futile to attempt to apply the findings from the study of one strain or species to any other strain or species. Just as Löhnis and Smith ('16) have pointed out the futility of attempting to apply the morphological findings in any particular medium or at any particular time to the group as a whole, it is well to emphasize the same with regard to physiological studies.

Since culture No. 62, apparently a strain of *Azotobacter chroococcum* and hence very closely related to culture No. 3a, seemed from the above-reported experiment, as well as from a number of unrecorded tests, to possess the ability to utilize a wider variety of salts of fatty acids than any other available culture, it was selected for the more intense studies reported in the next part of this paper. Also, even though aluminum acetate was undoubtedly more readily available to some strains of *Azotobacter* than the calcium salt, the latter served equally as well for culture No. 62; and since calcium salts have found a much wider use in biological studies than aluminum it seemed desirable to use the calcium salt, thus making any results that might be secured more comparable with those reported by others. In addition, calcium salts are somewhat more easily prepared than aluminum. Calcium was therefore used as the basic element in succeeding studies.

The question of the influence of the cation should certainly receive more study, and it is hoped that such studies may be continued in the near future. The data presented in table IX are only indicative of what may be expected.

Experiments with Culture No. 62

As previously mentioned, culture No. 62 probably belonged to the species *Azotobacter chroococcum*. Preliminary experiments indicated that it was a vigorously growing and strong nitrogen-fixing strain when supplied with a suitable form of organic material such as dextrose and certain of the lower fatty acids. The experiments conducted with this culture were all aerated.

Aeration was employed because the work of Mockeridge ('15) indicated that the rate at which certain organic materials were assimilated was extremely slow, long periods of incubation being necessary to insure appreciable utilization. Previous work had shown that the rate of growth, consumption of certain sugars, and fixation of nitrogen could be materially facilitated by drawing a current of air through the medium. It was hoped, by employing a similar method in these experiments, to shorten the time of incubation necessary to secure quantitative results of a definite character. Increased aeration unquestionably stimulated growth in many instances, but occasionally some difficulty was experienced in obtaining entirely satisfactory checking in quantitative nitrogen and volatile acid determinations following prolonged aeration, and in addition it was more difficult to maintain pure cultures. For these reasons the quantitative experiments in which *Azotobacter vinelandii* was employed were not aerated.

Utilization of formic acid.—Experiments were carried out in which formic acid was used as the sole organic constituent of the medium, but there was no indication of either growth, utilization of the acid, or fixation of nitrogen, and therefore the data are not recorded.

Utilization of acetic acid.—The data with regard to the utilization of acetic acid, recorded in table x, are quite conclusive in showing that the calcium salt of this acid is readily available to culture No. 62. Within seven days practically all the original 1.0 per cent of acid had disappeared, accompanied by abundant growth and a marked change in the reaction of the medium. In fact, it is probable that the hydroxyl-ion concentration was such as to inhibit further growth. Quite marked fixation of nitrogen was evident, but owing to an error in the method employed in the total nitrogen determinations in this experiment, the data are not recorded.

Utilization of propionic acid.—The ability of culture No. 62 to utilize readily the calcium salt of propionic acid is quite evident from the data presented in table xi. Within nine days practically all the acid had disappeared, and a marked increase in the hydroxyl-ion concentration and nitrogen content of the cultures had occurred.

TABLE X

UTILIZATION OF ACETIC ACID BY CULTURE NO 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	1059	——	Nitrogen fixation took place but owing to error in method the results were unsatisfactory.
2	Control 0	Sterile	7.0–7.4	1062	——	
3	2	Pure	7.0–7.4	831	229	
4	2	Pure	7.0–7.4	906	154	
5	4	(a)*	7.0–7.4	668	392	
6	4	(a)	7.0–7.4	654	406	
7	7	(a)	8.6–9.0	36	1024	
8	7	(a)	8.6–9.0	85	975	
9	9	Pure	9.0+	39	1021	
10	16	Contaminated	9.0+	48	1012	
11	16	Pure	9.0+	48	1012	
12	22	Contaminated	9.0+	124	936	
13	22	Pure	9.0+	91	969	

* Not tested.

TABLE XI

UTILIZATION OF PROPIONIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	958	—	—	—
2	Control 0	Sterile	7.0–7.4	952	—	—	—
3	Control 18	Contaminated	8.2–8.6	17	—	—	—
4	Control 18	Sterile	7.0–7.4	974	—	—	—
5	3	Pure	7.0–7.4	829	132	1.72	76
6	5	Pure	7.0–7.4	705	256	4.74	54
7	7	Pure	7.0–7.4	626	335	3.64	92
8	9	——	9.0+	21	940	——	——
9	12	Pure	9.0+	37	924	4.74	196
10	18	Contaminated	9.0+	17	944	——	——

In this experiment control flasks Nos. 1 and 2 were not aerated, while Nos. 3 and 4 were aerated under the same conditions and for as long a time as any of the inoculated flasks. The culture adjacent to control culture No. 3 foamed badly, resulting in the contamination of No. 3 with *Azotobacter;* therefore it is not considered in the quantitative calculations. It is evident,

though, from a comparison of control flasks No. 1 and No. 2 with No. 4 that no volatilization of the propionic acid took place. Therefore the decrease in the quantity of propionic acid that occurred in the presence of pure cultures must have been due to its assimilation by the organisms.

Utilization of normal butyric acid.—The data presented in table XII show that culture No. 62 is also capable of utilizing normal butyric acid in its metabolism. Again only seven days were required for almost complete assimilation of the acid present, with corresponding decreases in the hydrogen-ion concentration. The quantities of nitrogen fixed were also marked, as was the visible growth of the organisms.

TABLE XII

UTILIZATION OF NORMAL BUTYRIC ACID AND FIXATION OF NITROGEN BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	978	—	—	—
2	Control 0	Sterile	7.0–7.4	995	—	—	—
3	2	Pure	7.0–7.4	956	30	.09	334
4	2	Pure	7.0–7.4	878	108	1.25	86
5	5	Pure	7.0–7.4	706	280	2.73	102
6	5	Pure	7.0–7.4	669	317	4.15	76
7	7	Pure	7.0–7.4	439	547	2.59	212
8	12	Pure	9.0+	78	908	6.77	134
9	17	Contaminated	9.0+	61	925	4.95	186
10	17	Contaminated	9.0+	111	875	6.29	140

Utilization of iso-butyric acid.—Under the experimental conditions to which the cultures recorded in table XIII were subjected there was little or no indication that culture No. 62 could utilize iso-butyric acid in its metabolism. It is true that there was some decrease in the quantity of volatile acid present in the different cultures but the quantities were small. Besides, the non-inoculated sterile aerated controls, No. 2 and No. 3, showed practically the same decrease as inoculated flask No. 10, incubated the same length of time. In addition there was no perceptible change in the reaction, and the quantities of nitrogen fixed, if any, did not exceed the experimental error. Visible growth was also

questionable. It seems safe, therefore, to conclude that culture No. 62 could not utilize the calcium salt of iso-butyric acid under the conditions obtaining in these experiments.

TABLE XIII

UTILIZATION OF ISO-BUTYRIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
	Control					
1	0	Sterile	7.0–7.4	831	None	None
	Control					
2	17	Sterile	7.0–7.4	774	None	None
	Control					
3	17	Sterile	7.0–7.4	773	None	None
4	2	Pure	7.0–7.4	886	None	None
5	2	Pure	7.0–7.4	800	None	None
6	5	Contaminated	7.0–7.4	779	None	None
7	5	Pure	7.0–7.4	778	None	None
8	7	Contaminated	7.0–7.4	799	None	None
9	12	Pure	7.0–7.4	746	None	None
10	17	Contaminated	7.0–7.4	758	None	None

Utilization of normal valeric acid.—Not only was normal valeric acid not available to culture No. 62 but it actually was sufficiently toxic to kill all the introduced organisms within three days. This is the only instance in which any acid studied, other than formic, has actually killed the culture except when marked change in reaction occurred. There was of course no utilization of the acid or fixation of nitrogen. The slight loss of volatile acid previously referred to is evident in the data presented in table XIV.

Utilization of monohydrated valeric acid.[1]—The data presented in table XV with regard to this acid are inconclusive. There is a distinct loss of acid, evidently not due to its removal through aeration, because uninoculated aeration controls No. 3 and No. 4 incubated one day longer than the longest incubated inoculated flask showed no loss in volatile acid. On the other hand, there was no perceptible change in reaction, and the quantities of nitrogen fixed, if any, were too small to detect, there being no

[1] Mono- and trihydrated valeric acids were iso compounds made by Merck and Co.

TABLE XIV

UTILIZATION OF NORMAL VALERIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
1	Control 0	Sterile	6.6–7.0	992	None	None
2	Control 0	Sterile	6.6–7.0	992	None	None
3	3	Sterile	6.6–7.0	988	None	None
4	6	Contaminated	6.6–7.0	890	None	None
5	11	Sterile	6.6–7.0	990	None	None
6	11	Sterile	6.6–7.0	913	None	None
7	18	Sterile	6.6–7.0	973	None	None
8	18	Sterile	6.6–7.0	979	None	None
9	32	Sterile	6.6–7.0	935	None	None
10	32	Sterile	6.6–7.0	951	None	None

TABLE XV

UTILIZATION OF MONOHYDRATED VALERIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	1055	—	None
2	Control 0	Sterile	7.0–7.4	1058	—	None
3	Control 33	Sterile	7.0–7.4	1064	—	None
4	Control 33	Sterile	7.0–7.4	1066	—	None
5	3	Pure	7.0–7.4	1051	10	None
6	6	Pure	7.0–7.4	944	117	None
7	11	Pure	7.0–7.4	911	150	None
8	11	Pure	7.0–7.4	972	89	None
9	18	Pure	7.0–7.4	899	162	None
10	18	Pure	7.0–7.4	907	154	None
11	32	Pure	7.0–7.4	871	190	None
12	32	Pure	7.0–7.4	933	128	None

difference in the nitrogen content of flasks No. 11 and No. 12 and sterile controls No. 3 and No. 4.

Utilization of trihydrated valeric acid.—Here again the quantities of volatile acid not recovered and increases in total nitrogen were not sufficient to be regarded as significant. If the quantities of acid disappearing are based upon aerated control No. 3 no losses are evident. Unfortunately, this sample became con-

taminated, and conclusions based upon it would not be entirely valid. On the other hand, if the quantities of acid recovered from the various flasks are compared with those recovered from controls No. 1 and No. 2, analyzed at the beginning of the experiment, small losses of acid are evident. What has been said with regard to losses of acid is equally true of increases in nitrogen. It is preferred, therefore, to regard it merely as a questionable possibility that culture No. 62 assimilates this acid qualitatively. The quantitative utilization of this acid, as well as of the monohydrated sample, is certainly small, if it occurs at all, compared to that of some of the other acid studied.

TABLE XVI

UTILIZATION OF TRIHYDRATED VALERIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	784	Utilization questionable	Fixation questionable
2	Control 0	Sterile	7.0–7.4	785		
3	19	Contaminated	7.0–7.4	726		
4	3	Pure	7.0–7.4	719		
5	3	Pure	7.0–7.4	776		
6	7	Pure	7.0–7.4	756		
7	7	Contaminated	7.0–7.4	752		
8	13	Pure	7.0–7.4	766		
9	13	Pure	7.0–7.4	732		
10	19	Contaminated	7.0–7.4	743		
11	19	Contaminated	7.0–7.4	756		

Utilization of normal caproic acid.—The data presented in table XVII show beyond question that the calcium salt of normal caproic acid may serve as a readily available source of organic material for culture No. 62. The samples analyzed after seven days' incubation, while still containing large quantities of volatile acid, showed marked changes in reaction, appreciable losses of acid, and fixation of nitrogen. The samples analyzed after thirteen days still contained appreciable quantities of acid but the hydroxyl-ion concentration had probably reached a point where growth was inhibited. This is further indicated by the failure to detect further losses of acid in the flasks incubated for a longer time, No. 10 and No. 11.

TABLE XVII

UTILIZATION OF NORMAL CAPROIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	408	—	—	—
2	Control 19	Contaminated	9.0+	137	—	—	—
3	Control 19	Contaminated	7.0–7.4	359	—	—	—
4	3	Pure	6.6–7.0	351	57	1.02	56
5	3	Pure	7.0–7.4	374	34	.98	34
6	7	Pure	7.6–8.0	337	71	.50	142
7	7	Contaminated	7.8–8.2	260	148	2.16	69
8	13	Contaminated	9.0+	87	321	2.96	104
9	13	Contaminated	9.0+	75	333	Lost	—
10	19	Pure	9.0+	102	306	4.80	64
11	19	Pure	9.0+	109	299	—	—

Utilization of iso-caproic acid.—While the data presented in table XVIII may be regarded as inconclusive, they nevertheless indicate that iso-caproic acid is not available as an organic food for culture No. 62. There were slight losses of acid, but such losses were equally as marked from the non-inoculated, sterile, aerated controls as from any inoculated cultures. Further-

TABLE XVIII

UTILIZATION OF ISO-CAPROIC ACID BY CULTURE NO. 62

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed
1	Control 0	Sterile	7.0–7.4	560	Utilization questionable	Fixation questionable
2	Control 0	Sterile	7.0–7.4	533		
3	Control 19	Sterile	7.0–7.4	520		
4	19	Sterile	7.0–7.4	497		
5	3	Pure	7.0–7.4	526		
6	3	Pure	7.0–7.4	—		
7	7	Pure	7.0–7.4	522		
8	7	Pure	7.0–7.4	505		
9	13	Pure	7.0–7.4	517		
10	13	Contaminated	7.0–7.4	493		
11	19	?	7.0–7.4	513		
12	19	Sterile	7.0–7.4	518		

more, there was no appreciable changes in reaction or detectable increases in the nitrogen content.

Summary of experiments with culture No. 62.—Under the experimental conditions to which culture No. 62 was subjected in the experiments herein reported the calcium salts of formic and normal valeric acids were strongly germicidal. Similar salts of acetic, propionic, normal butyric, and normal caproic acids served as readily available sources of organic food. The other salts tested, namely, iso-butyric, mono- and trihydrated valeric and iso-caproic, were either not available or utilized very slowly and in small quantities.

Experiments with Azotobacter vinelandii

The culture of *Azotobacter vinelandii* used in these experiments (Culture No. 94) was obtained from N. R. Smith, of the Bureau of Plant Industry, U. S. Department of Agriculture. It grew vigorously upon soil-extract-mannitol agar, less vigorously upon beef-extract agar, and produced the typical green fluorescence upon the former medium. It also grew very abundantly in the liquid medium employed when dextrose, mannitol, and calcium salts of several of the fatty acids were supplied as the organic material.

Utilization of formic acid.—There was no evidence either in the qualitative or quantitative experiments of the ability of this organism to utilize calcium formate and therefore the quantitative data are omitted.

Utilization of acetic acid.—An examination of the data presented in table XIX should convince any one of the ability of this culture to utilize readily the calcium salt of acetic acid. A very marked change in the reaction, an almost complete disappearance of the acid accompanied by definite fixation of nitrogen, together with an abundance of visible growth, substantiate the above conclusions. In this experiment the hydroxyl-ion concentration evidently reached a point that could not be tolerated by the organisms, most of them being dead when the flasks incubated for the longer period of time were examined.

Utilization of propionic acid.—*Azotobacter vinelandii* can readily assimilate propionic acid under the conditions obtaining in the

TABLE XIX

UTILIZATION OF ACETIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	876	—	—	—
2	Control	Sterile	7.0–7.4	859	—	—	—
3	4	Pure	7.0–7.4	783	84	−.06	—
4	4	Pure	7.0–7.4	790	77	.20	384
5	9	Pure	7.0–7.4	667	200	−.20	—
6	9	Pure	7.0–7.4	575	292	.12	440
7	14	Pure	7.0–7.4	423	444	.50	888
8	14	Pure	7.0–7.4	389	478	.96	498
9	24	Pure*	9.0+	3	864	1.60	540
10	24	Pure*	9.0+	3	864	3.06	282
11	33	Sterile	9.0+	5	862	2.36	366
12	33	Pure*	9.0+	11	856	2.22	386

* Very few living organisms.

experiment reported in table xx. Within three weeks the original 1.0 per cent of acid had practically disappeared. The abundance of visible growth, the marked increase in hydroxyl-ion concentration, and definite increases in the nitrogen content of the cultures are additional proof of the ability of the organism to assimilate this particular acid.

TABLE XX

UTILIZATION OF PROPIONIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	1009	—	—	—
2	Control	Sterile	7.0–7.4	999	—	—	—
3	7	Pure	7.0–7.4	790	214	.32	668
4	14	Pure	7.0–7.4	575	429	1.46	194
5	23	Pure	9.0+	16	988	5.92	168
6	23	Pure	9.0+	45	959	6.06	158
7	30	Pure	9.0+	7	997	4.46	224
8	30	Sterile	9.0+	10	994	4.02	247
9	39	Sterile	9.0+	7	997	3.12	319
10	39	Sterile	9.0+	7	997	2.36	422
11	46	Sterile	9.0+	4	1000	2.22	450
12	46	Sterile	9.0+	6	998	3.12	319

Utilization of normal butyric acid.—Some irregularities were exhibited in the growth in different culture flasks containing

normal butyric acid and inoculated with *Azotobacter vinelandii* despite all efforts to make the duplicate flasks as nearly identical as possible. These irregularities are reflected in the quantity of acid unrecoverable, the changes in reaction, and in the increases in nitrogen content as recorded in table XXI. However, these data unquestionably show a ready utilization of this acid by the culture in question. The rapidity of disappearance of the acid is not as great as with acetic and propionic acids, but the increases in nitrogen per unit of acid assimilated are greater.

TABLE XXI

UTILIZATION OF NORMAL BUTYRIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	806	—	—	—
2	Control	Sterile	7.0–7.4	819	—	—	—
3	9	Pure	7.0–7.4	761	51	.12	424
4	9	Pure	7.0–7.4	750	62	.38	162
5	16	Pure	7.0–7.4	700	112	.76	148
6	16	Pure	7.0–7.4	700	112	.64	176
7	25	Pure	7.0–7.4	499	313	2.62	118
8	25	Pure	7.0–7.4	314	498	5.80	90
9	39	Sterile	9.0+	46	766	6.12	126
10	39	Pure	8.4–8.8	321	491	3.82	128
11	53	Pure	9.0+	51	761	6.12	124
12	53	Pure	8.8–9.2	411	401	3.56	112

Utilization of iso-butyric acid.—The data presented in table XXII indicate rather strongly that the calcium salt of iso-butyric acid is not nearly so readily available as is the corresponding salt of the normal acid. Even after forty-nine days over half of the original acid was still present, the reaction had changed only slightly, and the quantity of nitrogen fixed was small compared with that fixed where the normal acid was present. Besides, the visible growth (unmistakably present) was also small compared to that where acetic, propionic, or normal butyric acid was the source of organic food.

Utilization of normal valeric acid.—This acid apparently was not assimilated by *Azotobacter vinelandii* as readily as some of the lower members of the series. However, the figures presented in table XXIII show practically complete disappearance in flasks

TABLE XXII

UTILIZATION OF ISO-BUTYRIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	835	—	—	—
2	Control	Sterile	7.0–7.4	846	—	—	—
3	7	Pure	7.0–7.4	795	45	Lost	—
4	7	Pure	7.0–7.4	790	50	Lost	—
5	14	Pure	7.0–7.4	753	87	.26	334
6	14	Pure	7.0–7.4	Lost	—	.32	—
7	17	Pure	7.0–7.4	700	140	.12	—
8	17	Pure	7.0–7.4	728	112	.64	174
9	26	Pure	7.0–7.4	662	178	.64	278
10	26	Pure	7.0–7.4	634	206	.64	322
11	38	Pure	7.0–7.4	495	343	1.22	282
12	49	Pure	7.0–7.4	467	373	1.14	338

Nos. 9, 11, and 12. The more rapid assimilation in these instances, though, might have been partially due to the contaminating organisms. That there was, however, unmistakable utilization in the uncontaminated flasks, Nos. 7, 8, and 10, is proved by the decreased acid content, change in reaction, and increase in nitrogen content. Both the total quantity of nitrogen fixed and the relative quantity fixed per unit of acid assimilated were large, the latter being greater than for any lower member of the fatty acid series.

TABLE XXIII

UTILIZATION OF NORMAL VALERIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	892	—	—	—
2	Control	Sterile	7.0–7.4	883	—	—	—
3	7	Pure	7.0–7.4	810	77	.70	110
4	7	Pure	7.0–7.4	835	52	.70	74
5	14	Contaminated	7.0–7.4	770	117	.96	122
6	14	Contaminated	7.0–7.4	750	137	1.34	102
7	26	Pure	8.8–9.2	197	690	6.68	102
8	26	Pure	8.8–9.2	621	266	2.80	96
9	38	Contaminated	9.0+	54	833	6.76	122
10	38	Pure	7.4–7.8	414	473	6.56	72
11	49	Contaminated	9.0+	62	825	7.65	108
12	49	Contaminated	9.0+	60	827	11.98	70

Utilization of monohydrated valeric acid.—Growth in the presence of this acid was slow, and the total amount appeared to be much less than with the normal valeric or with the acids of smaller molecular weight. This was reflected in the rate and total disappearance of the acid as well as in the quantity of nitrogen fixed. It is also evident from the data recorded in table XXIV that slight, if any, change took place in the hydrogen-ion concentration. However, the amount of nitrogen fixed and acid consumed, coupled with the presence of visible growth, show conclusively that this acid can be assimilated by the culture in question, but probably not as readily as the other low molecular weight straight-chain, fatty acids, at least not under the conditions of these experiments.

TABLE XXIV

UTILIZATION OF MONOHYDRATED VALERIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	989	—	—	—
2	Control	Sterile	7.0–7.4	1021	—	—	—
3	9	Pure	7.0–7.4	831	192	.64	300
4	9	Pure	7.0–7.4	830	175	.76	230
5	21	Pure	7.0–7.4	816	189	1.40	136
6	21	Pure	7.0–7.4	841	164	1.52	108
7	28	Pure	7.0–7.4	685	320	2.62	122
8	28	Pure	7.0–7.4	714	291	1.52	192
9	38	Pure	7.0–7.4	637	368	2.04	180
10	38	Pure	7.0–7.4	712	293	1.66	176
11	49	Pure	7.0–7.4	631	374	1.90	196
12	49	Pure	7.0–7.4	663	342	2.16	158

Utilization of trihydrated valeric acid.—What was said with regard to the monohydrated valeric acid also applies to the trihydrated, except that the quantities of acid assimilated and the quantities of nitrogen fixed were smaller. No perceptible change took place in the hydrogen-ion concentration, and only one-fifth of the acid was not recoverable after seven weeks of incubation. These facts would indicate that this acid is assimilable by *Azotobacter vinelandii* with somewhat more difficulty than either the normal or monohydrated valeric acids. The data are presented in table XXV.

TABLE XXV

UTILIZATION OF TRIHYDRATED VALERIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	784	—	—	—
2	Control	Sterile	7.0–7.4	788	—	—	—
3	7	Pure	7.0–7.4	730	56	.38	148
4	7	Pure	7.0–7.4	753	33	.26	122
5	14	Pure	7.0–7.4	729	57	.26	218
6	14	Contaminated	7.0–7.4	714	72	.32	224
7	24	Pure	7.0–7.4	752	34	.50	68
8	24	Pure	7.0–7.4	708	78	.88	88
9	35	Pure	7.0–7.4	656	130	1.28	116
10	35	Pure	7.0–7.4	668	118	1.22	96
11	49	Contaminated	7.0–7.4	632	154	.76	202
12	49	Pure	7.0–7.4	628	158	.64	246

Utilization of normal caproic acid.—It would appear from the information recorded in table XXVI that normal caproic acid can be metabolized by *Azotobacter vinelandii* the most readily of any fatty acid tested. Within four days a very heavy growth was evident, half the acid added to the medium had disappeared, the reaction had become alkaline to thymol-blue, and marked fixation of nitrogen had taken place. Within nine days the volatile acid had reached the minimum recorded for any incubation period. Furthermore, the quantity of nitrogen fixed per

TABLE XXVI

UTILIZATION OF NORMAL CAPROIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	408	—	—	—
2	Control	Sterile	7.0–7.4	381	—	—	—
3	4	Pure	9.0+	267	128	3.92	32
4	4	Pure	9.0+	180	215	4.66	46
5	9	?	9.0+	39	356	5.86	60
6	9	Pure	9.0+	41	354	5.48	64
7	16	Pure	9.0+	32	363	6.06	60
8	16	Pure	9.0+	28	367	6.62	56
9	28	Contaminated	9.0+	27	368	5.28	70
10	28	Contaminated	9.0+	31	364	6.50	56
11	37	Contaminated	9.0+	35	360	8.34	82
12	37	Pure	9.0+	35	360	5.10	70

unit of acid assimilated was greater than for any other acid tested quantitatively. This would seem to indicate that increasing the size of the molecule does not necessarily decrease its availability.

Utilization of iso-caproic acid.—This acid was, according to the data presented in table XXVII, readily assimilated, though the rate of growth, acid utilization, and nitrogen fixation were not equal to the corresponding rates where normal caproic acid was the sole organic food supplied. Similarly, the ratio between acid consumed and nitrogen fixed was twice as wide as for the normal acid. The iso compound then, it would seem, is not only less readily metabolized but can also not be used as economically as the straight-chain molecule of this acid.

TABLE XXVII

UTILIZATION OF ISO-CAPROIC ACID AND FIXATION OF NITROGEN BY AZOTOBACTER VINELANDII

Flask No.	Days incubated	Purity	Approximate reaction expressed as P_H	Mgs. acid recovered	Mgs. acid utilized	Mgs. nitrogen fixed	Mgs. acid used per mg. nitrogen fixed
1	Control	Sterile	7.0–7.4	417	—	—	—
2	Control	Sterile	7.0–7.4	409	—	—	—
3	7	Pure	7.8–8.2	323	90	.50	180
4	7	Pure	7.8–8.2	272	131	.90	144
5	16	Pure	8.2–8.6	90	323	2.28	142
6	16	Pure	8.2–8.6	75	358	2.16	156
7	28	Pure	9.0+	23	390	2.54	154
8	28	Pure	9.0+	22	391	3.18	122
9	37	Pure	9.0+	77	336	3.70	90
10	37	Pure	9.0+	23	390	2.80	140
11	51	Sterile	9.0+	17	396	2.54	156
12	51	Pure*	9.0+	15	398	2.68	148

* Only four colonies developed on two plates.

Summary of experiments with Azotobacter vinelandii (culture No. 94).—*Azotobacter vinelandii* is capable of growing in a medium containing as the only organic material the calcium salt of the following fatty acids: acetic, propionic, normal butyric, iso-butyric, normal valeric, iso-valeric (monohydrated valeric and trihydrated valeric), normal caproic, and iso-caproic. This organism failed to grow under similar conditions in the presence of calcium formate.

The rate of growth varies with the different calcium salt added

to the medium, being very rapid with normal caproic and very slow with all of the iso acids tested. When growth takes place there is an increase in the nitrogen content of the medium and a decrease in the quantity of volatile acid.

The quantity of nitrogen fixed in the presence of any given acid corresponds more or less closely with the quantity of acid disappearing. The increase in nitrogen per unit of acid consumed by the organisms varies with different acids. If only normal acids are considered the quantity of nitrogen fixed per unit of acid decomposed increases as the molecular weight of the acid increases. Growth, disappearance of acid, and increase in nitrogen are not as rapid where iso acids are added to the medium as when the acid is a normal compound.

Experiments with Other Cultures

Since there were a number of instances in which the results secured with cultures No. 62 and No. 94 did not agree, it seemed desirable to extend somewhat similar tests to other cultures. In order to secure very active nitrogen-fixing strains for these tests the experiment recorded in table XXVIII was arranged. It was also hoped that this experiment would show to what extent vigorous growth in liquid media, utilization of dextrose, and fixation of nitrogen could be correlated.

Erlenmeyer flasks of 300 cc. capacity containing 1.0 per cent dextrose medium were prepared and inoculated heavily with the cultures indicated. One of the triplicate flasks was used for qualitative sugar tests. Growth observations were recorded from the remaining two flasks and after fifteen days' incubation the quantity of nitrogen fixed was determined.

Since the dextrose had completely disappeared in all but two instances and the original quantities in the various cultures were identical, the total nitrogen figures represent approximately the quantity of nitrogen fixed per 1000 mgs. dextrose consumed. It will be noted that with few exceptions the quantities of nitrogen fixed do not vary very widely. The smallest quantity fixed in any instance where complete disappearance of sugar had taken place was 2.64 mgs. while the largest was 11.46 mgs. Most of the cultures showed a fixation of about 8 to 10 mgs. per 1000

TABLE XXVIII

THE ABILITY OF VARIOUS AZOTOBACTER CULTURES TO GROW IN THE PRESENCE OF, AND TO ASSIMILATE, DEXTROSE, AND THEIR RELATIVE ABILITY TO FIX NITROGEN

Culture No.	Quantity of growth after varying periods of incubation			Presence of dextrose after varying periods of incubation		Nitrogen fixed per 100 cc. medium
	2 days	4 days	8 days	8 days	14 days	Mgs.
219	?	?	+	Abundant	0	8.14
187(a)	+	++++	++++	0	0	10.14
55	0	0	++	Abundant	0	9.16
209	++	++++	++++	0	0	9.92
C	++	+++	+++	0	0	10.62
94	+++	++++	++++	0	0	8.00
204	?	+++	++++	0	0	9.16
220	?	+	++	Abundant	0	9.10
15	?	?	?	Abundant	Abundant	2.64
19	+++	++++	++++	0	0	10.00
209	+	+++	++++	0	0	9.54
27	?	?	+	0	0	10.06
215(4)	++	+++	+++	0	0	8.66
II	++	+++	+++	Abundant	0	10.06
216	+++	+++	++++	0	0	9.04
144	?	?	++	0	0	10.38
16	?	?	++	Abundant	Abundant	2.74
103(4)	?	++++	++++	0	0	8.92
226(B)	+	+	++++	0	0	7.04
188	++++	++++	++++	0	0	8.28
86	+++	++++	++++	0	0	11.46
97	+++	+++	++++	0	0	9.30
95	++	+++	+++	0	0	7.06
B	?	?	+	Abundant	0	10.70
7	?	+	++	0	0	7.96
48	?	+	+++	Abundant	0	10.32
11(2)	++	+++	+++	0	0	8.92
56	?	+	++	0	0	9.16
S-2	0	?	+	Abundant	0	6.88
194	0	+	++	0	0	9.80
185	+	+	+++	0	0	8.08
165	?	+	++++	0	0	10.06
25	?	+++	+++	0	0	8.94
44	0	0	+	Abundant	0	8.46
I	0	+	+++	0	0	8.66
218	?	+++	+++	0	0	8.84
III	+	+++	+++	0	0	6.56
215(2)	+	+++	+++	0	0	6.24
178	0	?	++	0	0	10.62
198(1)	+	+++	+++	0	0	8.78
15(1)	0	0	+	0	0	7.64
Control	0	0	0	Abundant	Abundant	——
Control	0	0	0	Abundant	Abundant	——
Control	0	0	0	Abundant	Abundant	——

mgs. of dextrose or 100 to 120 parts of sugar consumed for each part of nitrogen fixed. This would indicate that the organic

food available, provided the particular culture in question is capable of utilizing it, is possibly of more significance in determining the quantity of nitrogen fixed than is the culture. There was, however, a rather marked variation in the rate at which various cultures consumed the dextrose.

Another very evident fact is that the quantity of visible growth cannot be taken as necessarily indicating the relative fixation of nitrogen. Cultures Nos. 219, 55, 220, 27, 144, B, 44, and 178 all showed a relatively small visible growth, yet they were among the most active nitrogen-fixing strains. Cultures Nos. 15 and 16 were evidently unable to utilize dextrose very readily. It is possible, though, that they may have fixed as much nitrogen per unit of dextrose consumed as any other culture.

From the cultures tested in the experiment just described nine, Nos. 7, C, 165, II, 188, 194, 187, 178, 218, and in addition R, were chosen for a comparative study of their ability to utilize the various acids studied in previous experiments. These particular cultures were selected both because of their active nitrogen-fixing ability and because they were secured from such widely varying conditions. Furthermore, they exhibited rather marked variations in cultural characteristics, indicating that they represented different strains or possibly species. In this experiment 100 cc. of the medium were placed in 750-cc. or 1000-cc. flasks, thus giving excellent aeration.

The rate of visible growth is indicated in table XXIX, while the quantities of acid consumed and nitrogen fixed are recorded in table XXX.

The data presented in tables XXIX and XXX tend to confirm the previous results secured from cultures No. 62 and 94 in that rather marked variations are exhibited in the ability of various cultures to grow in the presence of calcium salts of different organic acids as the only organic matter present.

All ten of the cultures readily assimilated dextrose, though culture No. II probably with somewhat more difficulty than the others. All grew in the presence of the calcium salts of acetic, propionic, normal valeric, normal butyric and normal caproic acids, the latter apparently being more easily metabolized than

TABLE XXIX

RELATIVE ABILITY OF TEN DIFFERENT CULTURES OF AZOTOBACTER TO GROW WHERE THE CALCIUM SALT OF DIFFERENT FATTY ACIDS WAS THE ONLY SOURCE OF ORGANIC FOOD

Culture No.	Growth in the presence of									
	Acetic acid	Propionic acid	Normal butyric acid	Iso-butyric acid	Normal valeric acid	Mono-hydrated valeric acid	Tri-hydrated valeric acid	Normal caproic acid	Iso-caproic acid	Dextrose
R (a)*	+++	++	0	?	++++	+	0	++++	0	++++
(b)	+++	+++	0	?	++++	+	0	++++	0	++++
(c)	+++	+++	0	?	++++	++	+	++++	0	++++
(d)	++++	+++	+	?	++++	++	+	++++	0	++++
(e)	++++	+++	+	?	++++	++	+	++++	0	++++
7 (a)	++	+	?	0	?	0	0	++	0	+++
(b)	++	+	+	?	?	0	0	++	0	+++
(c)	++	++	+	?	+	0	0	++++	0	+++
(d)	+++	+++	+	?	+++	0	0	++++	0	+++
(e)	++++	+++	+	+	+++	?	+	++++	0	+++
C (a)	+	+	0	?	+	?	?	+++	0	+++
(b)	+	+	?	?	+	?	?	+++	0	+++
(c)	+	+	+	?	++	?	?	+++	0	+++
(d)	+	++	++	?	+++	?	?	+++	0	+++
(e)	+++	++	+++	?	+++	?	?	+++	0	+++
218 (a)	++	+	0	0	++	+	0	++++	0	++++
(b)	+++	++	0	0	+++	+	?	++++	0	++++
(c)	+++	+++	+	+	++++	+	+	++++	0	++++
(d)	+++	+++	++	+	++++	+	++	++++	0	++++
(e)	++++	+++	++	+	++++	+	++	++++	0	++++
165 (a)	+	++	?	0	+	?	0	+	0	++++
(b)	+	+++	++	?	++	?	?	+	0	++++
(c)	+	+++	++	?	+++	+	?	++	0	++++
(d)	++	+++	++	+	+++	+	+	+++	0	++++
(e)	++	++++	++++	+	++++	+	+	+++	0	++++

TABLE XXIX (*continued*)

Culture No.	Growth in the presence of									
	Acetic acid	Propionic acid	Normal butyric acid	Iso-butyric acid	Normal valeric acid	Mono-hydrated valeric acid	Tri-hydrated valeric acid	Normal caproic acid	Iso-caproic acid	Dextrose
II (a)	?	0	0	0	?	?	0	+	0	?
(b)	+	0	0	0	?	?	0	+	0	+
(c)	+	?	0	0	+	?	0	+	0	+++
(d)	+	+	?	0	+	?	0	+	0	+++
(e)	++	++	+	?	+	?	0	+	0	+++
188 (a)	+++	+	+	+	+++	++	+	++	0	++++
(b)	+++	+	+	+	+++	++	+	+++	0	++++
(c)	+++	+	+	+	+++	++	++	+++	0	++++
(d)	+++	++	++	++	++++	++	++	+++	0	++++
(e)	++++	+++	++++	++	++++	++	++	+++	0	++++
194 (a)	+	+	?	0	++	0	0	+++	0	++++
(b)	+	+	?	?	++++	+	0	+++	0	++++
(c)	++	+	?	?	++++	+	0	+++	0	++++
(d)	+++	++	+	?	++++	+	0	+++	0	++++
(e)	++++	++	++	+	++++	+	?	++++	0	++++
187 (a)	+	+	?	+	+	?	?	+++	0	+++
(b)	+	+	+	+	++	?	?	+++	0	++++
(c)	+	+	+	+	+++	?	?	+++	0	++++
(d)	+	+	+	+	+++	+	?	+++	0	++++
(e)	+++	+	+++	+	+++	+	?	+++	0	++++
178 (a)	+	+	+	+	++	+	?	+++	0	+++
(b)	+	+	+	+	+++	+	?	++++	0	++++
(c)	+	+	+	+	++++	+	?	++++	0	++++
(d)	++	+	+	+	++++	+	?	++++	0	++++
(e)	++	++	+	+	++++	++	?	++++	0	++++

* (a) Observations made after 5 days incubation at 30° C.
(b) Observations made after 8 days incubation at 30° C.
(c) Observations made after 13 days incubation at 30° C.
(d) Observations made after 23 days incubation at 30° C.
(e) Observations made after 25 days incubation at 30° C.

TABLE XXX

GROWTH, UTILIZATION OF VARIOUS FATTY ACIDS, AND NITROGEN FIXED BY TEN DIFFERENT CULTURES OF AZOTOBACTER

Culture No.	Acetic acid	Propionic acid	Normal butyric acid	Iso-butyric acid	Normal valeric acid	Mono-hydrated valeric acid	Tri-hydrated valeric acid	Normal caproic acid	Iso-caproic acid	Dextrose
					Visible growth (35 days incubation)					
R	++++	+++	+	?	++++	++	+	++++	0	++++
7	+++	++++	++	+	+++	?	+	++++	0	+++
C	+++	++	+++	?	+++	?	?	+++	0	+++
218	++++	+++	++	+	++++	+	++	++++	0	++++
165	++	++++	++++	+	++++	+	?	++++	0	++++
II	++	++	+	?	+	?	0	+	0	+++
188	+++	+++	++++	++	++++	++	++	+++	0	++++
194	++++	++	++	+	++++	+	?	++++	0	++++
187	+++	+	+++	+	+++	+	?	+++	0	++++
178	++	++	+	+	++++	++	?	++++	0	++++
					Mgs. acid consumed					
R	883	618	276	71	378	152	—33	393	7	818
7	884	646	300	101	374	165	16	362	15	818
C	892	372	375	131	361	84	16	287	1	818
218	896	652	268	142	382	176	143	360	28	818
165	434	644	367	72	374	149	24	379	2	818
II	358	284	251	99	246	116	47	163	15	528
188	582	621	686	247	371	83	35	271	41	818
194	685	547	289	29	370	77	—51	247	1	818
187	385	386	189	247	375	143	7	393	21	818
178	379	391	308	70	365	57	—24	379	16	818

TABLE XXX (*continued*)

Culture No.	Acetic acid	Propionic acid	Normal butyric acid	Iso-butyric acid	Normal valeric acid	Mono-hydrated valeric acid	Tri-hydrated valeric acid	Normal caproic acid	Iso-caproic acid	Dextrose
					Mgs. nitrogen fixed					
R	3.19	2.29	2.29	.64	2.68	1.14	.26	3.06	.00	3.32
7	1.53	2.55	2.29	.77	3.82	1.02	.00	3.32	.00	4.72
C	2.68	1.53	2.04	.89	3.44	1.02	.77	4.08	.00	6.42
218	1.52	1.27	1.27	3.52	1.65	.77	1.14	3.82	.00	3.06
165	1.14	2.04	2.04	.51	2.55	.89	.12	3.44	.00	6.50
II	.77	1.02	1.02	.51	2.43	.64	.12	1.53	.00	5.10
188	1.53	3.32	3.82	.77	2.55	1.02	.00	4.08	.00	8.16
194	1.53	2.80	2.04	.64	1.53	1.27	.00	3.32	.00	6.42
187	1.40	1.40	1.32	.51	2.04	1.53	.00	3.82	.00	5.61
178	1.53	1.78	1.65	.77	1.78	.89	.00	3.95	.00	8.67
					Mgs. acid used per mg. nitrogen fixed					
R	277	270	121	109	141	133	—	128	—	247
7	578	253	131	131	98	162	—	109	—	173
C	333	243	135	147	115	82	—	70	—	127
218	585	513	211	—	231	229	—	95	—	267
165	381	315	180	141	147	167	—	111	—	126
II	463	278	246	194	101	181	—	107	—	103
188	367	187	179	321	143	81	—	66	—	100
194	448	231	141	—	241	61	—	74	—	127
187	275	276	143	484	184	93	—	103	—	164
178	248	220	187	91	205	77	—	96	—	94
Average	396	270	167	202	160	117		96		153
Mgs. nitrogen fixed per gm.	2.53	3.58	6.00	4.95	6.25	8.5		10.42		6.54
Mgs. nitrogen fixed per calory	.72	.72	1.01	.83	.93	1.27		1.45		1.73

any of the others, with valeric ranking next. It is quite evident, then, that increasing the size of the molecule does not decrease the availability. Not one of the cultures seemed capable of growing when the calcium salt of iso-caproic acid was the sole organic compound present. Growth with the calcium salts of iso-butyric or with the two hydrated valeric acids (the only organic materials present) was usually either very slow or absent. Culture No. 188 seemed to utilize a wider variety of acids with more ease than any other, its growth being recorded as + after three weeks with all the different acids except iso-caproic.

As previously pointed out, the quantity of visible growth is not necessarily associated with the quantity of nitrogen fixed or the quantity of organic material consumed. This is again evident if the visible growth as recorded in table XXIX is compared with the quantity of acid utilized and nitrogen fixed as recorded in table XXX.

The quantitative data presented in table XXX show very definitely that all ten of the strains of *Azotobacter* used in this experiment can utilize, as a source of organic food for nitrogen-fixing purposes, all the acids tested with the exception of tri-hydrated valeric and iso-caproic. Cultures C and 218 apparently utilized the trihydrated valeric acid. The iso compounds, though, cannot be assimilated as readily as the straight-chain molecules. Since this experiment could not be repeated no effort will be made to analyze critically the results secured with the individual cultures. It is quite evident that some of the cultures can assimilate certain acids very much more readily than can other cultures. Furthermore, some were capable of fixing very much more nitrogen per unit of acid with a given acid than were others.

The milligrams of acid consumed per milligram nitrogen fixed, the nitrogen fixed per gram acid, and the quantity of nitrogen fixed per calory of contained energy for the various acids were averaged and recorded at the end of table XXX. In a general way this summary agrees with the results secured with *Azotobacter vinelandii*. The quantity of nitrogen fixed per gram of acid metabolized increases as the molecular weight increases. In fact the increase in quantity of nitrogen fixed is more rapid

than the increase in heat of combustion, indicating a more efficient use of the energy contained in the larger molecules.

The conclusion seems justified, then, that the calcium salts of acetic, propionic, normal butyric, normal valeric, and normal caproic acids can be very readily assimilated by numerous strains of *Azotobacter*, while the ability to assimilate iso-caproic acid is rather limited among these organisms. The other acids tested, namely, iso-butyric and iso-valeric acids, while capable of being assimilated by most of the cultures tested, are certainly not as readily available as are acetic, propionic, normal valeric, and normal caproic.

As a further check on the relative availability of the various acids the protocol arranged in table XXXI was carried out experimentally. The acids used in this experiment were all from new lots and, because of limited quantities available, only approximately 0.5 per cent concentrations were used. Even with 0.5 per cent, large quantities of heptylic and caprylic acids remained undissolved in the culture medium. Erlenmeyer flasks of 300 cc. capacity containing 50 cc. of the medium and inoculated in duplicate served as cultures.

In addition to the fatty acids a number of polyhydric alcohols of varying molecular weights and some of identical molecular weights but varying configuration were used in order to see if similar variability in assimilability, as observed for acids, existed for alcohols.

Culture No. 94 again demonstrated its superiority over either No. 178 or No. 218 to utilize a variety of fatty acids, and also to metabolize more readily the alcohols. Both the latter cultures again failed to utilize any of the iso compounds readily and some not at all. These data, secured from entirely different batches of acids, tend to substantiate the observations noted from experiments recorded earlier in this paper. One new acid, di-methyl-ethyl-acetic, was added but apparently was not even assimilated by culture No. 94.

The same variability as regards the utilization of different acids by different strains of *Azotobacter* is also evident when the alcohols are considered. Of the eight polyhydric alcohols tested, only sorbitol and mannitol were readily metabolized by

TABLE XXXI

QUALITATIVE TESTS OF THE ABILITY OF AZOTOBACTER CULTURES NOS. 94, 178, AND 218 TO UTILIZE VARIOUS ORGANIC ACIDS AND ALCOHOLS

Organic material	Culture No. 94				Culture No. 178				Culture No. 218			
	6 days	10 days	19 days	39 days	6 days	10 days	19 days	39 days	6 days	10 days	19 days	39 days
Formic acid	0	0	0	0	0	0	0	?	0	0	0	0
Acetic acid	+++	+++	+++	+++	++	+++	+++	+++	+	++	++	+++
Propionic acid	+++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	+++
Lactic acid	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
Normal butyric acid	+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++	+++
Iso-butyric acid	++	++	+++	+++	?	?	?	+	?	+	+	++
Normal valeric acid	+++	+++	+++	+++	+	+	+++	+++	++	+++	+++	+++
Iso-valeric acid	0	+	++	+++	0	0	0	?	0	?	+	++
Normal caproic acid	+++	+++	+++	+++	+	++	++	+++	++	+++	+++	+++
Iso-caproic acid	+	+	+++	+++	?	?	?	?	0	0	0	?
Normal heptylic acid	+++	+++	+++	+++	?	+	++	+++	0	++	++	+++
Normal caprylic acid	+++	+++	+++	+++	?	?	?	+++	0	?	?	++
Di-methyl-ethyl-acetic acid	0	0	0	0	0	0	0	0	0	0	0	0
Ethylene glycol	0	0	0	+	0	0	0	0	0	0	0	0
Glycerol	+	++	++	+++	0	0	0	0	0	0	+	+++
Adonitol	0	0	0	+	0	0	0	0	0	0	0	0
Mannitol	+++	+++	+++	+++	0	+	+++	+++	0	0	++	+++
Dulcitol	0	0	0	+	0	0	0	0	0	0	0	0
Erythritol	+++	+++	+++	+++	0	0	0	0	0	0	0	0
Inositol	+++	+++	+++	+++	0	0	0	0	0	0	?	++
Sorbitol	+++	+++	+++	+++	+	++	+++	+++	0	++	+++	+++
Control	0	0	0	+	0	0	0	0	0	0	0	0
Control	0	0	0	+	0	0	0	0	0	0	0	0

all three cultures, the former more rapidly than the latter. Dulcitol, an isomer of sorbitol and mannitol, apparently cannot be utilized by any of the three cultures. Inositol, a six carbon hexahydric ring alcohol, was readily utilized by culture No. 94, with more difficulty by culture No. 218, and not at all by culture No. 178. The same was true of glycerol. The four carbon alcohol of this series, erythritol, was utilized only by culture No. 94. The two and five carbon numbers, ethylene glycol and adonitol, were apparently not available to any of the three cultures. This fact is rather interesting since Mockeridge obtained the maximum fixation in her experiments where ethylene glycol was the sole source of energy. Apparently the configuration of the molecule plays an important role in determining the ability of a given strain of *Azotobacter* to utilize a compound.

DISCUSSION

Attention has already been called to the salient facts brought out in the various experiments in the summaries accompanying the individual tables. In the limited discussion to follow an effort will be made merely to correlate these various points with the special object of trying to see if satisfactory answers to the original questions propounded in the introduction can be made.

In the first place, it is frankly admitted that certain procedures followed in some of the earlier experiments did not prove as satisfactory as had been hoped. This was true of the aeration in that the quantitative acid and nitrogen data did not, for some unknown reason, check as well as did later experiments. Because of these irregularities as much significance is not attached to the data secured in connection with those experiments as to those in which culture No. 94 was employed.

Secondly, such variation in the ability of different cultures to utilize different organic food substances was not anticipated or the experiments would have been confined entirely to identified cultures, thereby making it possible to compare results here reported with results obtained by other investigators. This desirability was realized in time to make use of known cultures in the major portion of these experiments, and data obtained with known organisms are regarded as much more significant.

In the third place the inadequacy of the data, in many or possibly all instances, from a quantitative point of view, is realized. It is believed, however, that in this respect they are equal or superior to any thus far reported. Where quantitative determinations, such as were employed in these experiments, are necessary the accumulation of mass data is a slow process. The limited data available are presented not as definite proof but rather as indicating certain tendencies, and it is hoped that others may see fit to conduct experiments along similar lines, thus bringing about the accumulation of sufficient data to justify definite conclusions.

It is desired to call attention again to, and to emphasize, the danger of applying the findings from a study of one strain of *Azotobacter* to other strains. If there is any one thing indicated by the experiments reported in this paper it is the marked variability in the metabolism of organic compounds of different strains or species in this group of organisms. This fact makes it rather difficult to compare results that have been reported from one laboratory with those from another, because frequently no indication whatsoever has been given as to the origin or identification of the culture studied. In the future much greater emphasis should be placed upon the identity of the culture being studied. For the reason just set forth it is believed that very little would be gained by a comprehensive review of investigations dealing with the utilization of various organic substances by this group of organisms.

A cursory perusal of the literature dealing with *Azotobacter* will impress one with the great variety of organic compounds that may be assimilated by these organisms. Also it brings out the wide variation in efficiency with which such compounds can be used when the quantity of nitrogen fixed in their presence is the criterion by which such efficiency is judged.

To illustrate the points suggested in the preceding paragraph the reader is referred to table XXXII. This table is an adaptation of one recently used by Bonazzi ('26) enlarged to include the work of Mockeridge and a few examples from the data previously presented in this paper.

Over fifty separate and distinct organic compounds are here

TABLE XXXII

RELATIVE FIXATION OF NITROGEN PER GRAM ORGANIC FOOD OBSERVED BY DIFFERENT INVESTIGATORS

Organic compound in medium	Mgs. nitrogen fixed per gram organic material						
	Löhnis and Pillai—impure		Hoffmann and Hammer		Krainsky	Mockeridge	Gainey
	$+CaCO_3$	$-CaCO_3$	Impure	Pure	Pure	Pure	Pure*
Polysaccharides:							
Starch	3.36	3.50			0.40	5.93	
Dextrine	7.18	7.58	1.72	13.40	1.20	6.62	
Inulin	7.72	7.58	1.35	10.85	5.80	9.76	
Gums:							
Gum arabic						6.13	
Gum tragacanth						9.13	
Sugars:							
Raffinose			2.23	5.35	1.60		
Saccharose	8.60	5.90	0.93	11.70	0.00	7.28	
Maltose	7.44	7.86	0.74		2.80	7.55	
Lactose	9.12	8.88	4.64	7.20	0.80	3.39	
Levulose	8.52	8.80	1.68	10.30	5.55	10.32	
Galactose	7.86	7.44	1.16	7.35	0.67	6.20	
Dextrose	4.62	4.36	1.65	8.95	1.35	6.57	8.85
Xylose	9.54	9.40		4.55		9.00	
Mannose				7.90			
Arabinose	7.62	7.34		10.00	0.60	9.28	
Alcohols:							
Mannitol	9.40	9.96	4.33	14.40	5.70	11.62	
Erythritol					0.00	4.88	
Glycerol	4.78	1.68		5.05	2.40	5.00	
Ethylene glycol						16.74	
Methyl					0.00	2.10	
Ethyl					1.00	4.02	
Propyl						9.02	
Iso-butyl						4.69	

TABLE XXXII (*continued*)

Organic compound in medium	Mgs. nitrogen fixed per gram organic material						
	Löhnis and Pillai—impure		Hoffmann and Hammer		Krainsky	Mockeridge	Gainey
	$+CaCO_3$	$-CaCO_3$	Impure	Pure	Pure	Pure	Pure*
Salts of fatty acids:							
Ca-formate						1.47	0.00
Ca-acetate					3.20	3.77	2.60
Na-propionate	1.10	0.96					
Ca-propionate					0.00	5.16	5.66
Ca-butyrate	0.02	0.16				6.08	6.52
Ca-butyrate (iso)							3.53
Ca-valeriate (normal)							10.71
Ca-valeriate (monohydrated)							5.99
Ca-valeriate (trihydrated)							7.49
Ca-caproate							17.87
Ca-caproate (iso)							7.57
Salts of other organic acids:							
Na-succinate	2.96	2.82					
Ca-succinate						8.60	
Na-citrate	1.42	1.00			1.60	6.44	
Ca-lactate	2.49	2.22				12.01	
K-oxalate	0.12	0.26					
Na-tartrate	5.06	2.82					
Ca-tartrate						4.54	
Ca-racemate						2.77	
Ca-malonate						5.32	
Ca-mucate						6.79	
Ca-fumarate						2.00	
Ca-maleate						1.88	
Ca-glycollate						1.75	
Ca-malate						5.19	

* Data for dextrose secured with culture No. 62 (*Azotobacter chroococcum*). Data for Ca-formate from large number of cultures including *Azotobacter chroococcum* and *Azotobacter vinelandii*. Other data are for culture No. 94 (*Azotobacter vinelandii*).

recorded as being assimilable by *Azotobacter*. Among these are representatives of classes of organic compounds possessing, in many instances, very few characteristics in common. Furthermore, the compounds here listed are only those reported by five of the many investigators in this field and are by no means intended to represent all compounds that have been tested and found capable of supplying the organic needs of members of the *Azotobacter* group of organisms. Among the carbohydrates are examples of polysaccharides, gums, and sugars. Among the latter are tri-, di-, and mono-saccharoses as well as hexose and pentose sugars. Alcohols are represented by mono-, di-, tri-, tetra-, and hexa-hydrox compounds, also by straight-chain and iso arrangements of the carbons, with the additional variations in polarity of inactive, dextro-, and levo-rotary molecules. There are also twenty-five salts of organic acids, including representatives of a number of dissimilar groups. There would seem to be no question, then, but that among the species of *Azotobacter* there are members capable of utilizing a very wide variety of organic compounds as the only organic requirement for the fixation of nitrogen. Not only is this true but the same strain of organisms can function as a nitrogen fixer when supplied with a wide variety of compounds.

When it comes to the comparative efficiency with which these various compounds can be used, measured by quantitative gains in nitrogen, the data are too inadequate to permit of drawing any very definite conclusions. The work with impure cultures would have to be eliminated from consideration. This leaves only a few compounds that have been tested by two or more investigators. Of these ten may be selected that were studied in common by Hoffmann and Hammer ('10), Krainsky ('08), and Mockeridge ('15). The quantity of nitrogen fixed per gram of material as reported by these investigators is indicated in fig. 3.

The data plotted may not be very accurate, since there is no indication of quantitative determinations of the residual organic material having been made, except that Mockeridge states that qualitative tests showed complete absence of the organic compound. Furthermore, one is forced to assume that the original

1.0 per cent of material added remained quantitatively unaltered during the process of sterilization, a condition that certainly might not obtain in all cases. In this connection it is believed that the policy followed in experiments herein reported, of making quantitative determinations on controls submitted to the same treatment as the cultures, is a much safer procedure for ascertaining the quantity of the organic compound available to the organisms.

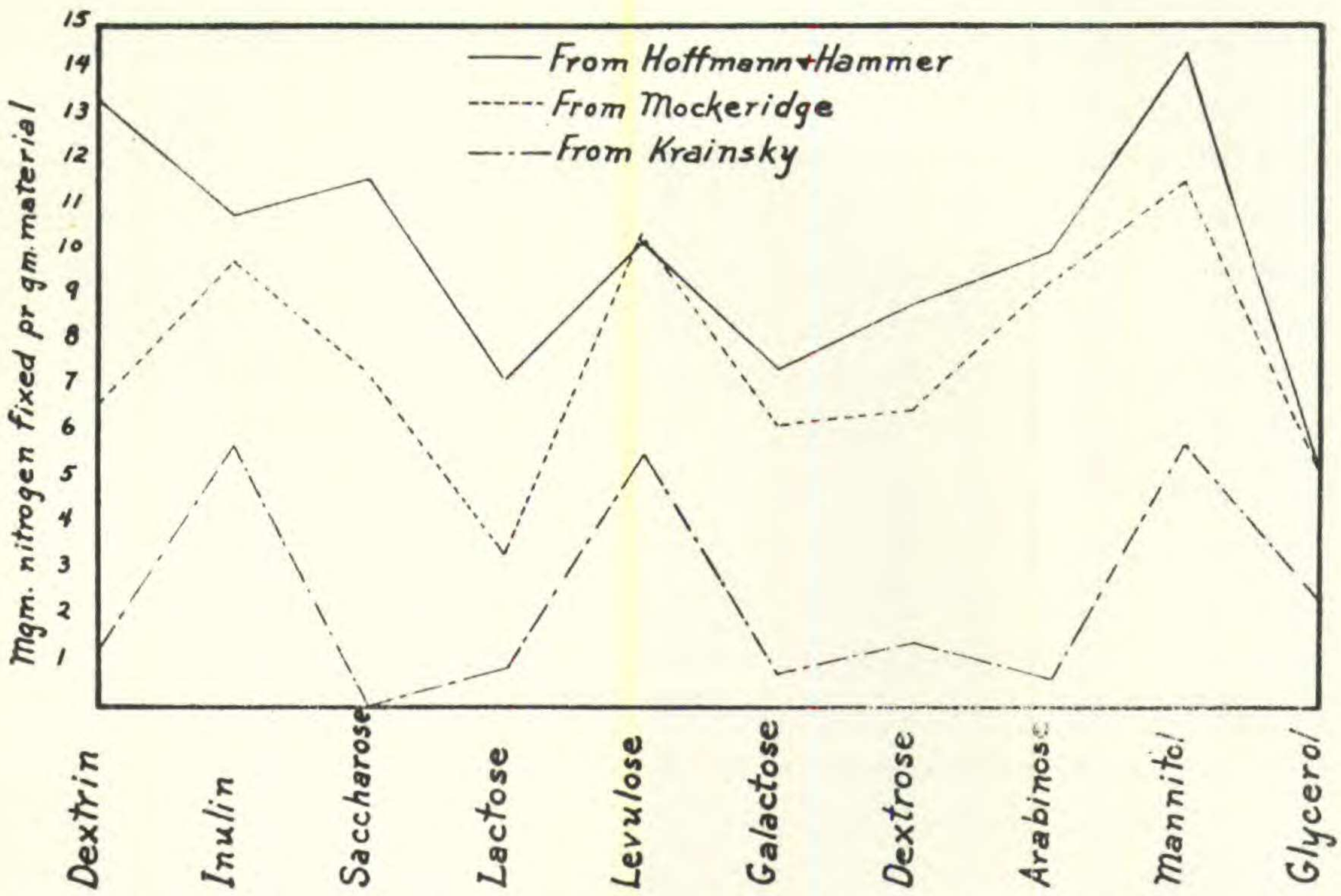

Fig. 3. Showing fixation of nitrogen per gram of organic material, secured by different investigators.

In spite of the numerous possibilities for errors the curves in fig. 3 show a marked qualitative similarity. The absolute quantities of nitrogen fixed per gram of material vary very widely. This might be taken to indicate a variation in the efficiency of the cultures, but, as mentioned in the preceding paragraph, it may merely mean that the organic material was not used up quantitatively in, for example, Krainsky's experiments.

If it is assumed that in all instances approximately the same quantity of organic material was available, and that it was used up quantitatively, then we would be justified in concluding that there are wide variations in the efficiency with which different

cultures utilize the same compounds and also a marked contrast in the efficiency with which the same culture used different organic materials in the fixation of nitrogen. There are strong indications, however, that different cultures may utilize many of the same compounds with approximately the same relative efficiency. Attention was called to this in connection with the experiment reported in table XXIII, in which out of forty cultures tested the quantity of nitrogen fixed, when dextrose served as the organic food, did not vary very widely in most instances.

The work here reported has been confined almost entirely to the salts of fatty acids, principally calcium salts. And attention may be called again to the possible marked effect that the cation may have upon the availability of the fatty acids, as indicated in table IX. It is necessary of course to use the salt of any acid to be tested in order to avoid the inhibiting effect of the hydrogen-ion concentration produced by the free acid. The only previously reported work with which these data can be compared directly is that of Mockeridge, recorded in table XXXII. That portion of Mockeridge's data dealing with fatty acids has been recorded in table XXXIII parallel with a summary of the data presented in this paper. In this table are recorded in parallel columns the molecular weights, heat of combustion, nitrogen fixed per gram of acid consumed, and nitrogen fixed per calory of heat energy contained in the material consumed.

Qualitatively, the only point of difference between the data presented here and those reported by Mockeridge is in the utilization of formic acid. In no instance have either quantitative or qualitative evidence of the growth of any culture of *Azotobacter* in the presence of a salt of formic acid been observed in this work.

Quantitatively, the data agree in showing that as the molecular weight or heat of combustion increases, the quantity of nitrogen fixed per unit of acid consumed increases. This is brought out clearly in fig. 4. There is, however, a difference, possibly significant, in the type of curves plotted from the two sets of data as shown in fig. 4 where the nitrogen fixed is plotted against molecular weight, or heat of combustion, since both increase arithmetically in the compounds as arranged in the figure.

TABLE XXXIII

MOLECULAR WEIGHT, HEAT OF COMBUSTION AND RELATIVE NITROGEN FIXED PER GRAM AND PER CALORY OF ORGANIC ACIDS BY CULTURE NO. 94 (AZOTOBACTER VINELANDII)

Acid	Molecular weight	Heat of combustion	Heat of combustion calories per gram	Mgs. nitrogen fixed per gram	Mgs. nitrogen fixed per calory	Mgs. nitrogen fixed per gm. (Mockeridge)	Mgs. nitrogen fixed per calory (Mockeridge)
Formic	46	61.7	1.34	0.00	0.00	1.47	1.10‡‡
Acetic	60	209.4	3.49	2.60*	0.74	3.77	1.09
Propionic	74	367.4	4.97	5.66†	1.14	5.16	1.04
Butyric	88	524.4	5.96	8.52‡	1.43	6.08	1.02
Valeric	102	681.6	6.68	10.71§	1.58		
Caproic	116	830.2	7.16	17.87§	2.49		
Iso-butyric	88	524.4††	5.96	3.53*	0.60		
Mono-hydrated-valeric	102	681.6††	6.68	5.99§	0.90		
Tri-hydrated-valeric	102	681.6††	6.68	7.49**	1.12		
Iso-caproic	116	830.2††	7.16	7.57*	1.06		
Dextrose	180	677.2	3.76	8.85	2.33	6.57	1.75

* Average of 5 determinations.

† Average of 3 determinations.

‡ Average of 8 determinations.

§ Average of 10 determinations.

** Average of 7 determinations.

†† Assumed to be same as for normal compounds.

‡‡ Recalculated for heats of combustion recorded in table. Mockeridge used different heat of combustion values.

This difference is shown more strikingly in fig. 5 in which the nitrogen fixed is plotted against a unit of energy contained in the compound.

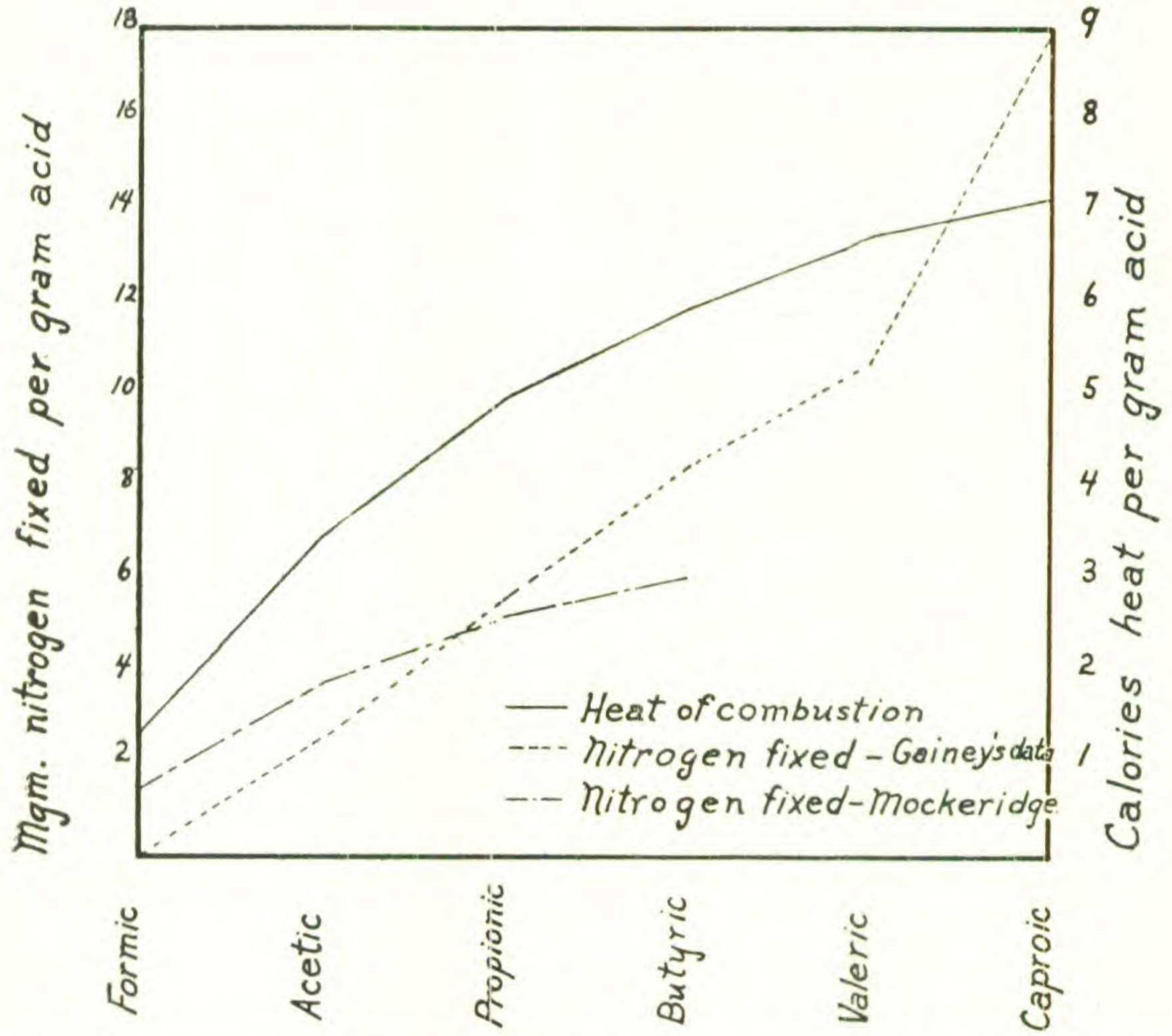

Fig. 4. Comparison between nitrogen fixation and heat of combustion per gram acid.

If the quantity of nitrogen fixed were proportional to the heat of combustion, then in fig. 4 all three curves should run parallel, while in fig. 5, the two lines should coincide and lie horizontal to the base line.

In fig. 4 the nitrogen-fixation curve plotted from Mockeridge's data tends to diverge from the curve for heat of combustion, and in fig. 5 it approaches the abscissa. Such curves would indicate that as the molecular weight increases the quantity of nitrogen fixed per unit of energy decreases. The decrease indicated in Mockeridge's data is very slight and is probably within the limit of error. On the other hand, the nitrogen fixation curve

in fig. 4 based upon new data starts well below the heat of combustion curve and actually crosses it with a marked upward tendency rather than a tendency to flatten out, while in fig. 5, starting on the base line, the curve continually, though not uniformly, rises from formic to caproic acid. Such curves indicate that as the molecular weight increases the organism is capable of utilizing the contained energy more efficiently. Atten-

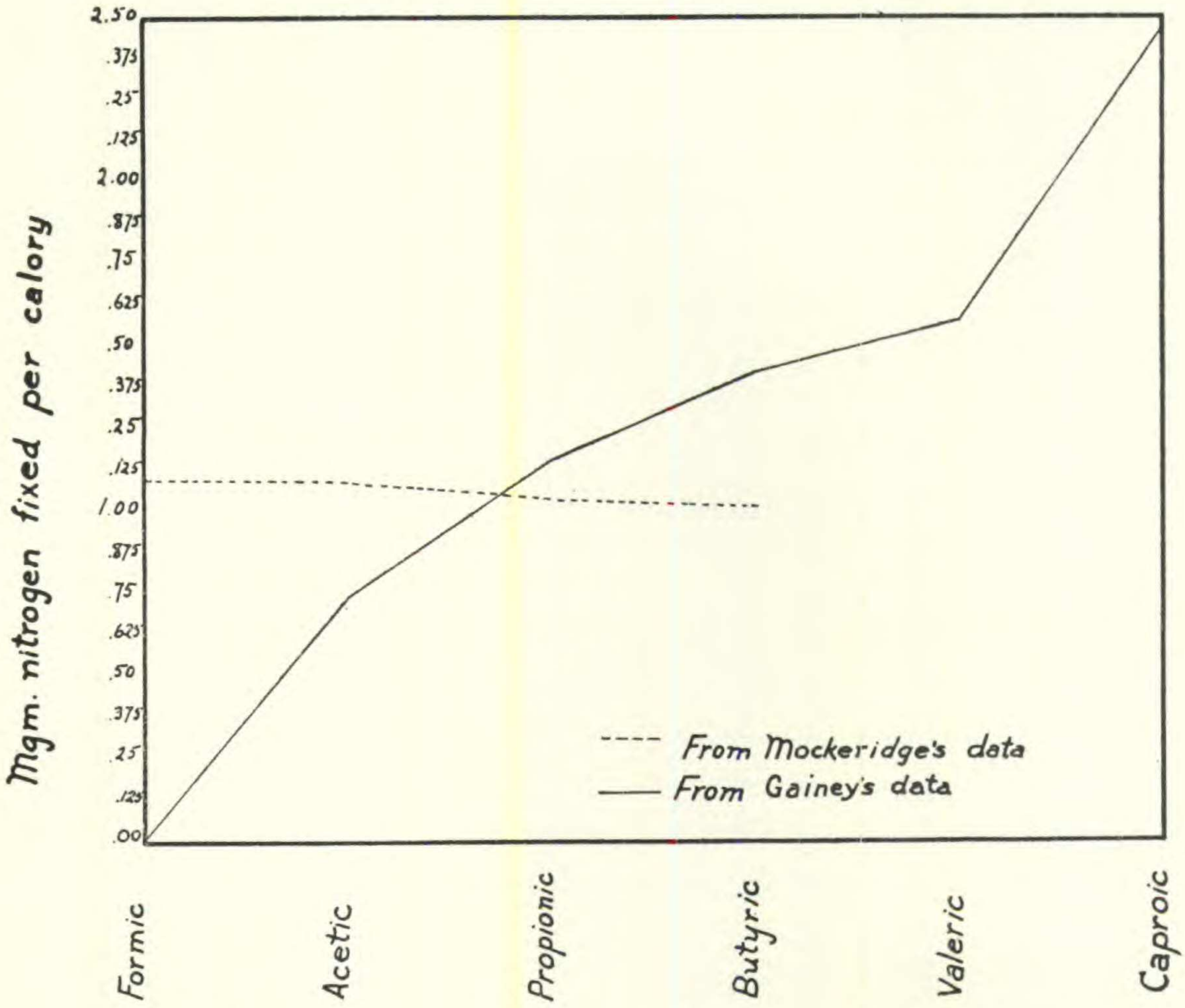

Fig. 5. Showing fixation of nitrogen per calory heat of combustion.

tion has previously been called to the fact that growth has been uniformly more rapid in caproic, and frequently in valeric acid, than in the acids of lower molecular weights.

The data serving as a basis for figs. 4 and 5 were secured from normal acids. In no instance has the iso acid appeared to be used as rapidly as the corresponding straight-chain compound. No explanation is offered as to the reason for this unless it is

that the straight-chain compounds are more frequently encountered by the organisms in nature and as a result they have become better adapted for metabolizing substances of this type. The data presented in table XXXIII indicate the same tendency on the part of *Azotobacter vinelandii* to utilize more efficiently, though not to the same degree, the iso acids of a higher molecular weight.

When the fatty acids are compared with dextrose and their efficiency measured in terms of nitrogen fixed, two points in connection therewith are worthy of note. If the nitrogen fixed per gram of material be used as a basis for comparison dextrose ranks about on a par with butyric acid, acetic and propionic being much inferior, while valeric and caproic, especially the latter, are superior. On the other hand, if the energy content be used as a basis for comparison, then dextrose ranks only slightly below caproic acid.

The data presented in table XXXIII are inconclusive in showing a close correlation between the energy content or heat of combustion of an organic compound and the efficiency with which *Azotobacter* can utilize it as the organic food required; nevertheless, there is more indication of a correlation when compared upon this basis than simply upon the actual weight of the material. There is an urgent need for much more data on the series of compounds herein reported, as well as other series, secured under carefully controlled quantitative conditions with definitely identified cultures, before any very definite conclusions can be drawn as to the energy relations concerned in the fixation of nitrogen by *Azotobacter*.

SUMMARY

The experiments reported in this paper have been carried out with the object of determining the relative ability of different cultures of *Azotobacter* to utilize qualitatively and quantitatively calcium salts of the different fatty acids up to and including six carbon atoms. Two cultures have been studied intensively, others to a less extent. The quantity of acid and total nitrogen present in the medium before and after varying periods of incubation were determined. The rapidity of growth was recorded,

as was also qualitative tests of changes in hydrogen-ion concentration.

The following is a summary of the more important tentative conclusions indicated by the limited experimental data secured:

(a) The various strains of *Azotobacter* behave quite differently with respect to their ability to utilize different fatty acids. Some cultures have been tested that seem rather limited in this respect, while others may utilize all of the acids tested.

(b) Individual cultures may vary widely not only in their ability qualitatively to utilize different acids but there may also be a marked difference quantitatively in this respect.

(c) The iso compounds are not as readily metabolized as are the normal.

(d) There is a marked tendency toward the reduction of the hydrogen-ion concentration in a medium in which an acid is utilized by *Azotobacter*. In some instances, apparently, this phenomenon may be responsible not only for the cessation of growth but actually for the death of the organisms.

(e) The quantity of nitrogen fixed is more or less proportional to the quantity of acid utilized.

(f) The quantity of nitrogen fixed per unit weight of acid consumed increases as the molecular weight or heat of combustion increases, provided comparisons are limited either to normal or iso compounds. There is some indication that the efficiency with which *Azotobacter* can utilize various acids, as measured by the quantity of nitrogen fixed, increases as the molecular weight increases, even when the comparison is based upon the energy content of the material utilized.

(g) The cation with which the acid is combined apparently plays a very important role in determining the ability of an organism to utilize the acid.

(h) The quantity of nitrogen fixed when various acids are utilized is more closely correlated with the energy content than it is with the actual weight of the material consumed.

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